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SOME DERIVATIVES OF CHOLESTEROL.

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(Received for publication, July 3, 1928.)

INTRODUCTION.

Because of its antihemolytic effect (1) attempts have been made to incorporate cholesterol (2) in certain serums and vaccines in order to prevent any hemolysis of red blood corpuscles when such solutions are injected. Owing to the insolubility of cholesterol, however, difficulty has been experienced in preparing solutions containing cholesterol. The object of the present work was to prepare a water-soluble derivative of cholesterol.

Cholesterol is a complex unsaturated secondary alcohol (3). In order to retain the antihemolytic effect the derivative chosen should not change the cholesterol molecule and should be hydrolyzable to cholesterol. The particular derivative sought was the *p*-aminobenzoate of cholesterol. It was hoped that salts of this amine would be water-soluble.

This derivative would also be of interest because of the possibility of local anesthetic action. Several of the simple esters of *p*-aminobenzoic acid (4) possess local anesthetic action, and the union of this grouping with cholesterol, a substance occurring in nervous tissue, might produce a compound possessing marked anesthetic action.

The same derivatives of dihydrocholesterol were also prepared in order to compare their properties with the corresponding compounds of the original unsaturated sterol.

Anderson (5) has found that cholesterol from different sources possesses slight differences in physical properties. The compounds prepared in the present work might also be of value in fractionating cholesterol itself and would be of service in identification and comparison of cholesterol with other reduced and unreduced sterols isolated from plants (6). It was for this reason

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that the preparation of the previously described benzoates was repeated, and the optical rotations determined, since the fractionation of sterols can be followed most easily by noting the change in specific rotation.

DISCUSSION.

The method chosen for the preparation of these derivatives consisted in condensing the sterol with *p*-nitrobenzoyl chloride followed by reduction of the resulting *p*-nitrobenzoate. Considerable difficulty was experienced in this reduction. Owing to its insolubility it could not be reduced by any of the usual reducing agents, iron, tin, and amalgamated zinc with hydrochloric acid all having no effect.

TABLE I.

Properties of Derivatives.

Substance	Cholesterol.		Dihydrocholesterol.	
	M.p. (corrected).	$[\alpha]_D^{25}$ $CHCl_3$.	M.p. (corrected).	$[\alpha]_D^{25}$ $CHCl_3$.
	°C.	degrees	°C.	degrees
Free sterol	149–150	−39.25	142–143	+26.35
Benzoate	144–145	−13.70	135.8–136.8	+21.40
p-Nitrobenzoate	188.8–189.8	−6.48	156.5–157.7	+20.05
p-Aminobenzoate	237.4–238.5	+3.61	191–192	+26.55

It was found that the *p*-nitrobenzoate could be easily reduced to the *p*-aminobenzoate by selective catalytic reduction. A solution of the nitro compound in ethyl acetate was shaken with hydrogen under pressure in the presence of the platinum oxide-platinum black (7) catalyst. By allowing the reduction to run for a short time, only the nitro group was reduced and the double bond in the sterol remained untouched.

The properties of the various derivatives prepared may be summarized in Table I.

From Table I it will be noted that the rotations of the cholesterol derivatives increase from the −39.25° of the free sterol to +3.61° of the *p*-aminobenzoate. The latter fact is most remarkable since from a consideration of the relative molecular weights the rotation of the amino compound should fall between that of the

benzoate and the *p*-nitrobenzoate. It was proved that this dextrorotation was not due to a reduction of the sterol molecule since saponification yielded free cholesterol with the same rotation as the original. A comparison of the rotations and melting points of the *p*-aminobenzoates of cholesterol and dihydrocholesterol also indicates that no reduction of the double bond had taken place.

The nitro group is reduced completely in 25 minutes, whereas about 4 hours are required to reduce cholesterol to dihydrocholesterol. The latter reduction is quite difficult and requires a larger amount of catalyst. In previous work on the reduction of sterols (8), ether, alcohol, or glacial acetic acid was used as the solvent. In the present work a careful study of different solvents led to the use of ethyl acetate. This solvent permits the reduction of 4 to 5 gm. of sterol in 4 hours, whereas glacial acetic acid requires 4 to 5 days for 1 gm. Moreover, the reduced sterol obtained from the reduction with ethyl acetate is *absolutely* negative to the Liebermann-Burchard reaction and possesses a rotation of $+26.35°$ which is considerably higher than that usually given. The rotations given in the literature range from $+23°$ to $+34°$. The present value agrees with that obtained by Doree (9), and since the product gives no Liebermann-Burchard reaction whatever, it is believed to represent the rotation of the completely reduced sterol. The higher value, $+34.00°$ of Boehm (10), is undoubtedly incorrect.

All the above aminobenzoates were insoluble in water and dilute hydrochloric acid. The hydrochlorides were prepared by passing dry hydrogen chloride into ether solutions of the bases. These salts were very insoluble so no hemolytic indices could be determined. Neither the amines nor their hydrochlorides exhibited any local anesthetic action at all, due undoubtedly to their insolubility.

EXPERIMENTAL.

The cholesterol used for the preparation of the following derivatives was purified by the method of Anderson (5). After two crystallizations from alcohol colorless plates were obtained. M. p. 149–50°, $[\alpha]_D^{25} = -39.25°$ in $CHCl_3$.

Preparation of Cholesteryl Benzoate.

5 gm. of dried cholesterol were heated with 2.5 gm. of benzoyl chloride at a temperature of 160° for 15 minutes. After cooling, the mass was disintegrated with water and then triturated with a large volume of alcohol which removes any unchanged cholesterol or benzoyl chloride. The insoluble portion, after filtration, was dissolved in ether and extracted twice with sodium bicarbonate solution and then with water. The ether solution was filtered, one-third its volume of alcohol was added, and the solution allowed to evaporate at room temperature. 4.5 gm. of colorless needles were obtained which melted at 144–145° after drying at 105° *in vacuo* over phosphorous pentoxide. $[\alpha]_D^{25} = -13.70°$ in $CHCl_3$.

Analysis. 0.1036 gm. substance: 0.3153 gm. CO_2, 0.0939 gm. H_2O.
$C_{34}H_{50}O_2$. Calculated. C 83.26 per cent, H 10.20 per cent.
Found. " 82.81 " " " 10.14 " "

The benzoate has been previously prepared by Doree (9), M. p. 150–151°, and by Obermüller (11), m. p. 145°. No rotations were given. Recrystallization of the above product did not change the melting point or rotation.

Preparation of Cholesteryl p-Nitrobenzoate.

A solution of 5 gm. of dried cholesterol in 15 cc. of toluene and 3.5 gm. of *p*-nitrobenzoyl chloride were heated in an oil bath at 165° for 15 minutes. The toluene was distilled off and 20 cc. of alcohol added to remove any unchanged *p*-nitrobenzoyl chloride. After the mixture was filtered, the precipitate was dissolved in benzene and washed with dilute sodium bicarbonate solution to remove any *p*-nitrobenzoic acid. After the solution was filtered, alcohol was added until a precipitate first appeared and the solution allowed to evaporate at room temperature until light yellow needles of the ester crystallized out. When dried it melted at 188.8–189.8° and possessed a rotation in $CHCl_3$ of −6.48°.

Analysis (Kjeldahl). 0.1543 gm. substance required 27.14 cc. 0.01 N HCl.
$C_{34}H_{49}O_4N$. Calculated. N 2.61 per cent. Found. N 2.46 per cent.

Doree and Orange (12) reported the *p*-nitrobenzoate as having a melting point of 185° but gave no rotation.

Preparation of Cholesteryl p-Aminobenzoate.

1 gm. of cholesteryl *p*-nitrobenzoate was dissolved in 150 cc. of ethyl acetate, 0.2 gm. of platinum oxide catalyst added, and the mixture shaken with hydrogen at 35 to 40 pounds pressure for 25 minutes in the apparatus described by Adams and Voorhees (13). After the platinum was filtered off and the ethyl acetate distilled off, the crude product was dissolved in benzene and washed with sodium bicarbonate solution and water. After the mixture was filtered, alcohol was added, which caused the *p*-aminobenzoate to separate out. After drying, it melted at 237.4–238.5° and its rotation was $[\alpha]_D^{25} = +3.61°$ in $CHCl_3$. A second preparation was made in order to check this rotation and it gave $[\alpha]_D^{25} = +3.50°$ in $CHCl_3$. It was absolutely insoluble in water and dilute hydrochloric acid.

Analysis (Kjeldahl). 0.1502 gm. substance required 29.50 cc. 0.01 N HCl.
$C_{34}H_{51}O_2N$. Calculated. N 2.77 per cent. Found. 2.75 per cent.

Saponification of Cholesteryl p-Aminobenzoate.

1 gm. of the above product was saponified by refluxing with alcoholic potassium hydroxide. After dilution with water, the cholesterol was extracted with ether. After the ether solution was filtered, the ether was distilled off and the residue recrystallized from alcohol. The product possessed the same melting point as the original cholesterol and gave a rotation in $CHCl_3$ of $-39.30°$.

Preparation of Cholesteryl p-Aminobenzoate Hydrochloride.

Dry hydrogen chloride was passed into an ethereal solution of cholesteryl *p*-aminobenzoate at 0°. After 2 minutes the precipitated hydrochloride was filtered off and heated *in vacuo* to remove any traces of solvent. It melted at 210–211° and was insoluble in water or dilute hydrochloric acid.

Analysis. 0.2280 gm. substance: 0.0599 gm. AgCl.
$C_{34}H_{52}O_2NCl$. Calculated. Cl 6.54 per cent. Found. Cl 6.49 per cent.

That hydrogen chloride had not been added to the double bond was shown by the fact that neither the benzoate nor *p*-nitrobenzoate gave a precipitate when an ethereal solution of these substances was treated with HCl at 0° for 2 minutes.

Preparation of Dihydrocholesterol.

5 gm. of cholesterol were dissolved in 160 cc. of ethyl acetate and 2 gm. of platinum oxide catalyst added. The mixture was shaken with hydrogen at 35 to 40 pounds pressure for 4 hours. After the platinum was filtered off and the solution concentrated slightly, the dihydrocholesterol separated out in colorless crystals which were then recrystallized twice from alcohol. It melted at 142–143° and its rotation in $CHCl_3$ was +26.20°. In ether $[\alpha]_D^{25}$ = +31.30°. A second preparation was made to check the above rotation. It gave +26.35° in $CHCl_3$.

Analysis. 0.1181 gm. substance: 0.3605 gm. CO_2, 0.1294 gm. H_2O.
$C_{27}H_{48}O$. Calculated. C 83.50 per cent, H 12.37 per cent.
Found. " 83.25 " " " 12.26 " "

A sample of dihydrocholesterol was prepared by catalytic reduction in glacial acetic acid as a solvent. This product melted at 141–142° and gave the following rotations: $[\alpha]_D^{25}$ = +23.17° in $CHCl_3$ and +28.05° in ether. These values agree with those previously reported by Anderson (5), von Fürth and Felsenreich (14), Willstätter and Meyer (15), and Ellis and Gardner (16). This product, however, was incompletely reduced as shown by the fact that it gave a positive Liebermann-Burchard reaction.

Derivatives of Dihydrocholesterol.

The benzoate, *p*-nitrobenzoate, *p*-aminobenzoate, and the hydrochloride of the latter were prepared by exactly the same procedure as outlined above for cholesterol.

Dihydrocholesteryl Benzoate.

Colorless needles; m. p. 135.8–136.8°; $[\alpha]_D^{25}$ = + 21.40° in $CHCl_3$.
Analysis. 0.1223 gm. substance: 0.3700 gm. CO_2, 0.1153 gm. H_2O.
$C_{34}H_{52}O_2$. Calculated. C 82.92 per cent, H 10.57 per cent.
Found. " 82.51 " " " 10.55 " "

Dihydrocholesteryl p-Nitrobenzoate.

Light yellow needles; m. p. 156.5–157.7°; $[\alpha]_D^{25} = +20.05°$ in $CHCl_3$.

Analysis (Kjeldahl). 0.1342 gm. substance required 24.80 cc. 0.01 N HCl.

$C_{34}H_{51}O_4N$. Calculated. N 2.60 per cent. Found N 2.58 per cent.

Dihydrocholesteryl p-Aminobenzoate.

Very light yellow amorphous product, insoluble in water and dilute hydrochloric acid. M. p. 191–192°; $[\alpha]_D^{25} = +26.55°$ in $CHCl_3$.

Analysis (Kjeldahl). 0.1483 gm. substance required 28.15 cc. 0.1 N HCl.

$C_{34}H_{53}O_2N$. Calculated. N 2.76 per cent. Found. N 2.65 per cent.

Dihydrocholesteryl p-Aminobenzoate Hydrochloride.

White amorphous product insoluble in water. M. p. 182.5–184.5°.

Analysis. 0.2212 gm. substance: 0.0581 gm. AgCl.

$C_{34}H_{54}O_4NCl$. Calculated. Cl 6.53 per cent. Found. Cl 6.49 per cent.

SUMMARY.

1. The benzoates, *p*-nitrobenzoates, *p*-aminobenzoates, and the hydrochlorides of the *p*-aminobenzoates of cholesterol and dihydrocholesterol have been prepared and their properties studied.

2. The best solvent for use in the catalytic reduction of cholesterol is ethyl acetate.

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A NEW PROCESS FOR THE SEPARATION OF THE VITAMIN FRACTION FROM COD LIVER OIL.

By JOSEPH KEATS MARCUS.

(From the Research Laboratories of Pitman-Moore Company, Indianapolis.)

(Received for publication, July 19, 1928.)

When, about 8 years ago, the unsaponifiable matter of cod liver oil was shown to be the fraction carrying its vitamin content, the separation of the unsaponifiable matter from the oil became a matter of great scientific importance, for this fraction constitutes a starting material in attempts to isolate the vitamins A and D. Furthermore, a potent cod liver oil concentrate is of great value in therapeutic and prophylactic medicine.

The method used in the original preparation (1) of the vitamin fraction from cod liver oil was an adaptation of the classical procedure for determining unsaponifiable matter in a fat. The cod liver oil is saponified with alcoholic alkali and the resulting alkali soap after solution in water is extracted with ethyl ether. The unsaponifiable matter is found in the ether extract. The apparent simplicity of this procedure is belied by difficulties in its practical operation, as is soon evident when the preparation of more than a small amount of concentrate is attempted. Chief among these difficulties are: (1) the excessive volumes of water and alcohol necessary in order to prevent emulsions, (2) the inflammability of the extraction solvent used, (3) the possibility of spontaneous explosions (2) due to the accumulation of ethereal peroxides.

As was to be expected, this unsatisfactory state of affairs gave rise to investigations of other solvents which could be used in place of ethyl ether for the extraction of the alkali soaps. But no success was reported (2). However, so great was the urge to get away from the use of ether that indirect methods of winning the unsaponifiable fraction from cod liver oil were sought. Three new processes were the outcome of these efforts. (1) Takahashi (3) saponifies cod liver oil with alkali, converts the alkali soap to an alkali

earth soap, and then extracts the unsaponifiable matter from the latter with alcohol. (2) Zucker (4), applying a method used for the determination of sterols in oils, first subjects cod liver oil to extraction with alcohol. The alcohol removes the unsaponifiable matter together with some of the oil. The alcoholic extract, after removal of the alcohol, is saponified with alkali, the alkali soap is converted to an alkali earth soap, and the latter extracted with acetone to remove the unsaponifiable matter. (3) Funk and Dubin (5) attain a similar end by first extracting the cod liver oil with glacial acetic acid. The glacial acetic acid extract contains the unsaponifiable matter and part of the oil. After removal of the acetic acid the residue is saponified with alkali and extracted with ether as in the classical method for unsaponifiable matter.

From the standpoint of the number of operations involved, none of these three new methods[1] equals the classical ether extraction procedure for simplicity. Apart from the disadvantage obvious in a multiplicity of operations in any process, particularly is simplicity a prime desideratum in the preparation of a cod liver oil vitamin concentrate because of the lability of these vitamins, particularly vitamin A.

Accordingly we decided to reopen the investigation of the alkali soaps of cod liver oil and their behavior with organic solvents. Our aim was to find an organic solvent which would make possible the simplicity in operation desirable in a process for the extraction of the fat-soluble vitamins without the drawbacks attending the classical ether extraction method.

The alkali soaps of cod liver oil when completely dissolved in water produce emulsions of varying degrees of permanence with all the common organic solvents. Only in the presence of considerable amounts of alcohol is successful extraction with ethyl ether possible, and even more alcohol is required with petroleum ether. On the other hand, when the alkali soaps are dried, the solvents either emulsify with them or dissolve them in considerable amounts.

However, we have found that there is a middle ground where the alkali soap may be obtained in the form of a magma by limit-

[1] The Takahashi procedure removes only vitamin A with the unsaponifiable matter. Zucker removes only vitamin D. Funk and Dubin extract both vitamins A and D.

ing the amount of water and alcohol present during saponification. This soap magma possesses the interesting property of being susceptible to successful extraction with ethylene dichloride. The ethylene dichloride is heavier than the soap and separates quickly and sharply as a clear lower layer. Ethylene dichloride possesses quite ideal properties for our purpose. It is practically non-inflammable. It forms no explosive products. It has a very low solubility for water. And whereas its boiling point is high compared to ether, yet its distillation *in vacuo* takes place at a temperature below the boiling point of ether.

The alkali soap magma may be produced by saponifying the cod liver oil in homogeneous solution with alcoholic alkali, as in the usual manner, then distilling off the alcohol, and finally adding a limited amount of water. However, it was found that a much more direct and convenient method can be used for producing the soap magma. It is based on the principle of "cold saponification," by which is meant a saponification of an oil with concentrated aqueous alkali which proceeds as an exothermic reaction. We have found that cod liver oil undergoes such an exothermic reaction when treated with concentrated aqueous alkali in the presence of a small amount of alcohol (less than 1 per cent on the basis of the cod liver oil taken). The exothermic reaction will not take place without the alcohol, but once the small amount of alcohol is added the reaction is vigorous and is completed in a short time.

"Cold saponification" has been applicable hitherto only for oils of the cocoanut oil group. On the basis of our finding with cod liver and other oils, the addition of a small amount of alcohol may make it possible to extend this method to all the oils.

Details of the two methods are given below.

Process 1. Cold Saponification.

260 gm. of potassium hydroxide were dissolved in 250 cc. of water. This solution while warm was added to 1000 cc. of cod liver oil. The mixture was vigorously stirred and 10 cc. of alcohol were added. The reaction started at once. The mass stiffened and the temperature rose. After the mass had cooled, 90 cc. of water were added and the whole vigorously agitated with ethylene dichloride. The ethylene dichloride layer separated below the soap which is lighter. It was clear yellow and contained only a small amount of dissolved

soap. This small amount of soap was removed by simple agitation with anhydrous calcium chloride. Agitation with water will accomplish the same end, but subsequent calcium chloride treatment is necessary to dry the ethylene dichloride. The ethylene dichloride solution was now distilled *in vacuo*. The residue was a yellow-orange semisolid substance that responded strongly to the well known color tests for vitamin A. By the well known biological assays it was demonstrated that the concentrate possessed the full vitamin A and D potency of the original oil from which it was extracted. All the operations, of course, were carried out under anaerobic conditions wherever possible in order to safeguard vitamin A from oxidation.

Process 2. Saponification in Homogeneous Solution.

When stirring arrangements are not available for saponifying the cod liver oil as in the first method, saponification may be brought about in homogeneous alcohol solution. The alcohol is then distilled off until the soap is in the form of a magma. The proper amount of water is added and then the extraction with ethylene dichloride is carried out as above.

An example of this method is as follows: 250 gm. of potassium hydroxide are dissolved in a mixture of 840 cc. of alcohol and 100 cc. of water. 1 liter of cod liver oil is added and a homogeneous solution results. The temperature of the solution is maintained around 60° for about 40 minutes. The alcohol is distilled over, preferably *in vacuo*. About 620 cc. of alcohol are recovered in the distillate. After being cooled, the soap magma in the distilling flask is treated with 300 cc. of water and extracted with ethylene dichloride. The ethylene dichloride extract is somewhat turbid, owing to some dissolved soap. The soap is removed by a water wash, the ethylene dichloride is dried with calcium chloride, and the ethylene dichloride is distilled *in vacuo* as above.

Comparing Processes 1 and 2 we see that the amounts of alcohol and water present in the soap at the time of extraction vary. In Process 1 there is present 1 per cent alcohol and 34 per cent water on the basis of cod liver oil treated, whereas the figures for Process 2 are about 22 per cent alcohol and 40 per cent water. It is interesting to compare these figures with those for the classical ether extraction procedure in which there is present in the soap

solution at the time of extraction 200 per cent alcohol and 1000 per cent water on the basis of cod liver oil used. The great improvement over the ether procedure is obvious.

The greater amount of alcohol used in Process 2 over that in Process 1 doubtless accounts for the greater turbidity of the ethylene dichloride extract of the former. This is probably due to the ethylene dichloride taking up more alcohol and thus increasing its solubility for the soap.

Noteworthy, also, is the fact that ethylene dichloride is heavier than the alkali soap. The advantage of this property is that the soap layer need never be removed from the extractor in the course of successive extractions. It is the ethylene dichloride which is drawn off. Thus is effected a simplicity in operation which is of moment where the labile vitamine A is concerned. In the ether extraction process the soap layer is heavier than the extraction solvent.

None of the other common organic solvents tried either structurally related to ethylene dichloride or otherwise, including ethyl ether, can take the place of ethylene dichloride with the same success. They all form emulsions of varying degrees of permanence. Thus the success of the new process resides not only in the physical condition of the soap with its water and alcohol content but also in the specific properties of ethylene dichloride.

The applicability of the new process for the extraction of unsaponifiable matter from oils and fats in general is being investigated. Preliminary experiments with a few oils show that similar results are obtained as with cod liver oil. Also, the adaptability of the new process as an analytical method for the determination of unsaponifiable matter in fats is being investigated.

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ANTIRICKETIC SUBSTANCES.

VIII. STUDIES ON HIGHLY PURIFIED ERGOSTEROL AND ITS ESTERS.*

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It is generally understood that the principal sterol of fungi is ergosterol. This substance was extracted from ergot by Tanret (1908), who purified it with notable success. Several later workers have employed yeast as a more convenient source, but the melting points and rotations of their preparations are not in agreement and do not equal the criteria established by Tanret (Table I). Failure to attain satisfactory purification is due to the instability of the ergosterol, and especially to the difficulties inherent in separating mixed sterols by crystallization.

The following procedure was designed to prepare ergosterol of the greatest possible purity for research on antiricketic activation. Crude yeast sterol was obtained from the unsaponifiable fraction of the fat of the common yeast, *Saccharomyces cerevisiæ*. Examination of many commercial batches of this product showed it to consist of at least three sterols, separable with difficulty. One is a low melting sterol, perhaps identical with the zymosterol of MacLean (1928). Another melts above 240°, and may be named cerevisterol without too great offense to etymology. Ergosterol itself is the main component. Imperfectly separated mixtures of these three sterols melt anywhere from 80–240°, and have apparently been mistaken for pure ergosterol in the literature.

Investigation of the effectiveness of several common solvents and many solvent mixtures in the purification of ergosterol led us to adopt alcohol-benzene for recrystallization. 50 gm. of the

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crude sterol were dissolved in 1 liter of a boiling mixture of 95 per cent alcohol (2 volumes) and benzene (1 volume). The hot solution was rapidly filtered through hardened paper on a silvered vacuum funnel, and cooled slowly until a slight turbidity just became perceptible in the supernatant fluid above the ergosterol crystals (beginning precipitation of cerevisterol or zymosterol).

TABLE I.

Showing the Melting Point and Specific Rotation of Yeast Ergosterol as Reported by Different Investigators.

Investigator.	Melting point.	Specific rotation in chloroform.	Remarks.
	°C.		
Gérard (1895)	135–136	$[\alpha]_D = -105°$	
Hinsberg and Roos (1903)	159		
Neville (1913)	145–147		$[\alpha]_D = -75.54°$ in unspecified solvent.
Meisenheimer (1915)	156–157		
MacLean and Thomas (1920)	154	$[\alpha]_D^{18} = -117°$	
Windaus and Grosskopf (1922)	154		
Reindel, Walter, and Rauch (1927)	160–161	$[\alpha]_D = -117°$	
Reindel and Walter (1928)	160		Purified *via* acetate.
MacLean (1928)	158.5	$[\alpha]_D = -100°$*	Yeast presumably the source. Chloroform presumably the solvent for $[\alpha]_D$.
Windaus and Borgeaud (1928)	163	$[\alpha]_D = -130°$	
Bills and Honeywell	168	$[\alpha]_D^{20} = -132°$	Nearly anhydrous.
	175		Semihydrous.
	183		Very hydrous.

* Calculated from MacLean's value, $[\alpha]_{5461} = -130°$. We determined that $[\alpha]_{Hg} \div [\alpha]_D = 1.297$ for ergosterol in $CHCl_3$ at 20.0°.

Ergosterol came out of the liquid as acicular leaflets, while most of the pigment and contaminant sterols remained in solution. The ergosterol crystals after being washed with a little 95 per cent alcohol and pressed dry were almost snow-white, and under the microscope appeared homogeneous.

On account of the instability of ergosterol all purification treat-

ments must be conducted as expeditiously as possible, in the absence of strong light, and with minimum heating. Merely boiling ergosterol in such a low boiling solvent as acetone results in some decomposition, this being greatly accelerated by traces of catalysts, such as charcoal.

The best criterion of the purity of ergosterol is its optical rotation, as determined in chloroform solution. Since the hydrated form of ergosterol gives turbid solutions in chloroform (Tanret), it is necessary to dehydrate all test samples. Our procedure in every case was to subject the sample to the vacuum of a Hyvac pump for 1 hour at room temperature and then for 10 minutes at 80°. The accuracy of our polarimetric determinations was checked by testing a Bureau of Standards sucrose sample, our findings agreeing with those of the Bureau to within 0.01°.

The once recrystallized ergosterol, dehydrated, exhibited $[\alpha]_D^{20} = -129°$. The particular batch of crude sterol from which this ergosterol was prepared gave $[\alpha]_D^{20} = -120°$. Obviously one recrystallization effected the removal of a considerable amount of impurity of low optical activity. The ergosterol was recrystallized a second time from 500 cc. of alcohol-benzene, giving snow-white crystals, $[\alpha]_D^{20} = -132°$; yield, 31 gm. A third and a fourth recrystallization effected no significant change in specific rotation, and so it appeared that the limit of purification by this means had been reached. However, this is no proof that the ergosterol was pure. One recalls the failure of several recent attempts to separate cholesterol-ergosterol mixtures by crystallization alone.

In the fractionation of sterols by crystallization better results are sometimes obtained by working up the esters. In this way, *via* the acetate, Reindel, Walter, and Rauch (1927) and Reindel and Walter (1928) effected partial purification of a crude yeast ergosterol. With us another ester, the isobutyrate, was more satisfactory than the acetate. Ergosteryl isobutyrate can be crystallized in remarkably large, perfect prisms, which present a smaller area for surface contamination than either the acetate or free sterol.

Another sample of anhydrous ergosterol, partially purified, $[\alpha]_D^{20} = -128°$, was converted into isobutyrate as follows: 10 gm. of sterol and 20 cc. of isobutyric anhydride, *free from chloride*, were heated in an oil bath for 10 minutes at 135–140°. The pale

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yellow product crystallized solid on cooling. The hard mass was carefully comminuted in 100 cc. of 80 per cent acetone, allowed to stand for 1 hour, filtered, washed thoroughly with 80 per cent acetone, and dried in air at room temperature. The snow-white product was recrystallized from boiling ether-acetone (1:3) by refrigeration overnight. The resultant massive crystals, 1 to 2 cm. long, were washed with a little pure acetone and dried.

The limit of purification was reached by a second recrystallization, and the ester was then saponified: 7.5 gm. of ergosteryl isobutyrate were added to 500 cc. of boiling 97 per cent alcohol containing 11 gm. of purified NaOH. The heating was continued for 5 minutes after the last crystal dissolved. The solution was cooled to 35°, diluted with 125 cc. of water, and refrigerated for 6 hours. The precipitate was filtered with suction, washed with 80 per cent alcohol, and then with 65 per cent alcohol until the filtrate was neutral to litmus. The fine, colorless product was recrystallized twice from alcohol-acetone (1:1). Yield, 5.6 gm. of snow-white leaflets.

The ergosterol thus purified *via* the isobutyrate exhibited the same specific rotation, −132°, as that purified directly by crystallization from the mixture of alcohol and benzene. This fact may be regarded as evidence, not alone conclusive, that the ergosterol was pure.

We have already mentioned the two contaminant sterols associated with yeast ergosterol. Both are less soluble than ergosterol in most solvents, and it might therefore be suspected that the exceptionally high specific rotation, −132°, was due to admixture with them. However, this is not the case, for the specific rotation of the contaminants is low. A specimen of zymosterol, m.p. 80°, gave $[\alpha]_D^{20} = +7°$ in chloroform, and a cerevisterol preparation, m.p. 240°, gave $[\alpha]_D^{20} = -49°$. Since both specimens were but sparingly soluble in chloroform, and neither was carefully purified, the rotations given are only suggestive. They prove, nevertheless, that the high rotation found for ergosterol is not due to either contaminant.

The value, $[\alpha]_D^{20} = -132°$, is the highest ever ascribed to ergosterol from yeast, and it is identical with the figure given by Tanret (1908) for his most carefully purified anhydrous ergosterol from ergot. In ergot the principal contaminant is the feebly levorota-

tory fungisterol, which Tanret found *more* soluble than ergosterol. Tanret's description of the properties of fungisterol clearly differentiates it from either of the contaminant sterols of yeast. It is probable, therefore, that both his preparation of ergosterol and ours are pure, because they exhibit the same high specific rotation, and are surely not likely to be similarly contaminated.

The identity of our ergosterol with Tanret's is further indicated by crystallographic comparison. Tanret reported the data on crystal structure given by his associate, Lacroix, who examined a specimen in some detail. For the examination of our product we thank Dr. J. T. Singewald and Mr. E. H. Watson of the Johns Hopkins University. Specially large crystals were obtained from a solution of the pure ergosterol in ether-acetone (1:3). These were found to consist of elongated six-sided prisms, the angles adjacent to the long sides measuring about 127°, and the angles between the short sides 106°. The extinction is parallel, the sign positive, and the plane of the optical axes transverse to the length. It is somewhat uncertain whether the crystals are monoclinic or orthorhombic.

It would seem that the recent discoveries in the relation of ergosterol to vitamin D have all been made with ergosterol of questionable purity. Until now only Tanret had prepared ergosterol free from contamination, but this was done years before the current studies on activation. We desired to establish whether the antiricketic and spectrographic properties of ergosterol are characteristic of the pure sterol, or are associated with a contaminant. In cholesterol, as we have previously shown (1928), these properties are partly, though not wholly, due to contamination. While the details of our studies on activation will be published later, it is sufficient to state now that pure ergosterol, unlike zymosterol and cerevisterol, is highly activatable. We have prepared irradiated specimens so potent that 1 part in more than a billion parts of diet gave evidence of action in rat rickets. Spectrographic studies by Mr. W. M. Cox, Jr., of this laboratory, have shown that the absorption bands, found by other investigators, in ordinary ergosterol are in fact given by pure ergosterol, and not by zymosterol or cerevisterol.

To Tanret's good description of the properties of ergosterol may be added some notes of our own. Ergosterol does not exhibit

mutarotation in chloroform. However, on standing a week or more, the chloroform reacts with the ergosterol, the solution gradually darkening. Exposed to daylight, the solution deteriorates rapidly, becoming brown in a few hours. The reaction with chloroform is quite different from the yellowing that occurs in inert solvents.

Pure ergosterol is slightly less unstable than ergosterol of ordinary quality. Nevertheless it gradually becomes yellow or greenish yellow when kept at room temperature (25–30°). Exposure to light accelerates the yellowing. At 0° in the dark a sample of ordinary ergosterol exposed to the air has remained colorless for a year.

The specific rotation of ergosterol varies considerably with the temperature, this effect being relatively greater for $[\alpha]_{Hg}$ than for $[\alpha]_D$. The following data were calculated for 20.0° and 25.0°, when $c = 1$ in chloroform:

$$[\alpha]_D^{20} = -131.6°$$
$$[\alpha]_D^{25} = -130.5°$$
$$[\alpha]_{5461}^{20} = -170.6°$$
$$[\alpha]_{5461}^{25} = -165.6°$$

Crystals of anhydrous ergosterol and ergosteryl esters become highly electrified by friction with metal. Several cholesteryl esters and ethers behave similarly.

Ergosterol in benzene solution is destroyed by the clay catalyst, floridin. The decomposition occurs at room temperature and with far greater rapidity than Bills (1926) noted in the catalytic degradation of cholesterol.

The melting point of ergosterol as ordinarily reported is of little significance. Early in our work we made hundreds of melting point determinations, but we were seldom able to obtain the same value twice in succession, even on samples presumably homogeneous. The explanation of this anomaly was found in the fact that the melting point of ergosterol is exceedingly sensitive to differences in the water content of the sample.

In sealed capillary tubes *totally submerged* in the heating bath hydrated pure ergosterol (dried in moist air at 30°) began to sinter at 165°, melted clear at 175°, solidified at 174°, remelted clear at

178°, and resolidified at 174°. The same preparation, dehydrated, sintered at 160°, melted clear at 168°, solidified at 167°, remelted clear at 171°, and resolidified at 167°. This preparation was not indeed completely anhydrous. In fact, we never made a preparation that was, because we preferred to leave the last traces of moisture that were tenaciously held, rather than to risk decomposition in the drastic treatment necessary to remove them. After exposure to moisture, this product again gave the melting points of the hydrated form. When the dehydrated ergosterol was wetted with 25 per cent alcohol, and then dried only enough so that it was not sensibly wet, although fully hydrous, it melted clear at about 183°. When dehydrated ergosterol was examined in sealed capillary tubes only partially immersed in the heating bath, the m.p. was observed to be about 2° lower than when the tubes were completely immersed—an effect due obviously to the last traces of moisture being driven into the cool part of the capillary.

The foregoing melting points are corrected averages for a group of consistent readings. In sealed tubes ergosterol suffers little decomposition, for it can be remelted several times without altering the remelting and solidification points. Tanret also had trouble with his melting points, obtaining different values by different methods. He attributed the difficulty to oxidation, but more probably it was a matter of hydration. One may state that pure ergosterol melts at from 166–183°, according to the degree of hydration.

Tanret described four esters of ergosterol—formate, acetate, propionate, and *n*-butyrate, and noted that they crystallize water-free. Reindel, Walter, and Rauch prepared the benzoate. We again made the acetate and benzoate, and also three new esters—isobutyrate, isovalerate, and cinnamate. Each of our preparations was recrystallized to the limit of purification. Only the cinnamate exhibits colors on cooling from the molten state, whereas most of the esters of cholesterol give color play. Ergosterol esterifies readily, the cinnamate apparently forming at considerably greater velocity than cholesteryl cinnamate under the same conditions.

Ergosteryl Acetate.—The method of preparation is analogous to that for isobutyrate. Yield, 6.4 gm. from 10 gm. of ergosterol.

Snow-white leaflets from ether-acetone (1:3). $[\alpha]_D^{25} = -90°$, when $c = 1$ in $CHCl_3$. This ester sinters at 178°; liquifies at 179°; melts clear at 181° (corrected).

Ergosteryl Isobutyrate.—The method of preparation was as above described. Yield, 7.5 gm. from 10 gm. of ergosterol. Massive snow-white crystals from ether-acetone (1:3). $[\alpha]_D^{25} = -84°$, when $c = 1$ in $CHCl_3$. This ester sinters at 145°; becomes a viscous turbid liquid at 148°; becomes a thin turbid liquid at 159°; melts clear at 162°.

Ergosteryl Isovalerate.—The method of preparation was analogous to that for isobutyrate, but recrystallizations were from pure acetone. Yield, 5.4 gm. from 10 gm. of ergosterol. Very fine snow-white needles, odorless. $[\alpha]_D^{25} = -82°$, when $c = 1$ in $CHCl_3$. This ester sinters at 135°; becomes a viscous turbid liquid at 138°; becomes a thin turbid liquid at 157°; melts clear at 160°.

Ergosteryl Benzoate.—Prepared by the usual method with benzoyl chloride in cold pyridine. Recrystallizations from acetone-benzene 4:1. Yield, 5.4 gm. from 10 gm. of ergosterol. Small needles after prolonged standing in solvent at 0°; highly electrifiable. $[\alpha]_D^{25} = -177°$, when $c = 1$ in $CHCl_3$. This ester sinters at 162°; liquifies at 164°; melts clear at 168°.

Ergosteryl Cinnamate.—10 gm. of dehydrated ergosterol and 10 gm. of cinnamoyl chloride were heated at 170° for 5 minutes. The product was dissolved in ether at room temperature, and filtered through hardened paper. To the clear solution 1.5 volumes of 95 per cent alcohol (2850 cc.) were added. The solution after standing overnight at −16° was filtered, the cinnamate leaflets washed with a little pure acetone, and dried. Recrystallizations were made from ligroin *purified by permanganate.* Yield, 6.8 gm. of colorless, silvery, lustrous leaflet masses. $[\alpha]_D^{25} = -22°$, when $c = 1$ in $CHCl_3$. This ester sinters at 175°; liquifies at 177°; melts clear at 179°. On cooling, the melt solidifies at from 145–140° with a beautiful play of colors.

SUMMARY.

1. Crude yeast sterol is a mixture of ergosterol and two other sterols. One is dextrorotatory and possibly identical with Mac-

Lean's zymosterol. The other, named cerevisterol, is levorotatory, and melts above 240°.

2. The ergosterol of yeast is identical with that of ergot, and has been isolated in pure form. Purification was accomplished in two ways—by recrystallization from an exceptionally effective solvent mixture (alcohol-benzene (3:1)), and by saponification of purified ergosteryl isobutyrate.

3. Pure ergosterol exhibits in chloroform $[\alpha]_D^{20} = -132°$ and $[\alpha]_{5461}^{20} = -171°$. The melting point is of little significance as an index of purity, for it varies from 166–183°, according to the hydration of the sample.

4. The spectrographic and physiological properties associated with ergosterol of ordinary purity are exhibited by ergosterol free from contaminants.

5. Three new esters, ergosteryl isobutyrate, isovalerate, and cinnamate, have been prepared and purified.

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THE ESTIMATION OF SILICA IN TISSUES.

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The estimation of silica in animal organs by the gravimetric method is attended with considerable difficulty. Large amounts of tissue are necessary, and ashing by the method of Stolte (1), or even with the aid of an electric muffle furnace is a tedious process requiring several hours for completion. Analysis of the ash for silica in the conventional way also takes considerable time, so that an estimation is a fairly lengthy process.

Isaacs (2) seemed to have supplied the need for a rapid and easily manipulated procedure in his colorimetric method for the estimation of silica in tissue. Discordant and inaccurate results, however, attended the use of Isaacs' method in this laboratory. The colors produced in both standard and test solutions were not proportional to the concentrations, and in many cases, even with the utmost care, duplicate estimations did not check. In the case of tissues high in calcium, there was invariably formed, when the test solutions were heated in the water bath, a heavy precipitate of calcium molybdate. This precipitate was a source of constant trouble, as the test solutions had to be filtered before they could be read, and in several instances it was thought that some of the blue color was being carried down by the calcium molybdate. At any rate the results obtained by this method did not agree with gravimetric analyses done as a check on the same tissues, and the discrepancy was largest where the precipitate in the solution had been heavy.

In the present paper a micro method is proposed which makes use of the intense yellow color formed when a silicate solution is treated with ammonium molybdate and sulfuric acid. The method is quick, and easily applied, and the proportionality of

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color produced in test solutions prepared from sodium silicate and from ashed tissue holds over a wide range of concentration.

The production of yellow silicomolybdic acid formed the basis for Dienert and Wandenbulcke's (3) colorimetric method for estimating small amounts of silica in water, and was used by Atkins (4) and by Thresh (5) in their studies of the silica content of natural waters.

The yellow color is of the same tint as that of a dilute solution of picric acid, so that an artificial standard is possible. An artificial standard is preferable to a sodium silicate standard since the latter tends to deposit silica, and is difficult of standardization.[1] The picric acid standard remains unchanged for at least 2 months, and can be made from any good brand of C.P. picric acid without recrystallization.

Phosphate, reckoned as P_2O_5, gives less than a tenth of the color given by an equal weight of SiO_2. In some cases where the phosphorus content is low a fairly reliable estimate of the silica present may be got without precipitation of the phosphate. But for its accurate determination, the removal of phosphate is necessary, and is accomplished by adding magnesia mixture to the solution of the ash, and filtering off the magnesium ammonium phosphate.

That the silica is not partially precipitated along with the phosphate, but is quantitatively estimated by the method, is shown by the following experiments.

Experiment I.

The silica of a mixed silicate phosphate solution is recovered in the filtrate after precipitation of the phosphate.

Solution 1.—10 cc. of sodium silicate solution containing 1 mg. of SiO_2, were diluted to 40 cc. with water, treated with 2 cc. of 10 per cent ammonium molybdate and 4 drops of 50 per cent (by volume) sulfuric acid, and made to 50 cc.

Solution 2.—1 mg. of SiO_2 and 2 mg. of P as K_2HPO_4 in 35 cc. of water were treated with magnesia mixture and ammonia.

[1] Isaacs used silicate solutions made of highly diluted water glass, which he standardized against a solution containing a known weight of SiO_2, which had been fused with sodium carbonate and dissolved in water.

After being filtered and washed, the solution was neutralized, treated with ammonium molybdate and sulfuric acid, and made to volume.

Solution 3.—1 mg. of SiO_2 and 5 mg. of P were treated in the same way.

These solutions had the same depth of yellow color.

Experiment II.

Silica added to tissue is quantitatively recovered as shown by difference. Several different tissues were analyzed according to the method before and after the addition of silica.

Lung 1 gave 11.40 mg. of SiO_2 per gm. of dried tissue, and 16.40 mg. after the addition of 5 mg. of SiO_2 per gm.; difference 5 mg.

Lung 2 gave 6.80 mg. of SiO_2 per gm. of dried tissue, and 11.76 mg. after the addition of 5 mg. of SiO_2 per gm.; difference 4.96 mg.

Liver 1 gave 0.80 mg. of SiO_2 per gm. of dried tissue, and 1.28 mg. after the addition of 0.5 mg. of SiO_2 per gm.; difference 0.48 mg.

Egg gave 0.2 mg. of SiO_2 per 10 gm. of white, and 0.69 mg. after the addition of 0.5 mg. of SiO_2; difference 0.49 mg.

Experiment III.

Values given by the colorimetric procedure check closely with those obtained by the gravimetric method. The results of several analyses are as follows:

Comparison of SiO_2 Values Given by Colorimetric and Gravimetric Methods.

Sample 1. 0.2 gm. of silicotic Lung 409 analyzed colorimetrically gave 63.0 mg. of SiO_2 per gm. of dried tissue.
5.0 gm. of the same ashed in an electric muffle furnace and analyzed gravimetrically gave 0.3180 gm. of SiO_2 or 63.6 mg. of SiO_2 per gm. of dried tissue.

Sample 2. 0.25 gm. of silicotic Lung 410 gave by the colorimetric method 60.0 mg. of SiO_2 per gm. of dried tissue.
5.0 gm. of the same gave by the gravimetric method 0.3035 gm. of SiO_2 or 60.7 mg. of SiO_2 per gm. of dried tissue.

Sample 3. 0.1 gm. of silicotic Lung 411 gave by the colorimetric method 29.6 mg. of SiO_2 per gm. of dried tissue.
8.5 gm. of the same gave by the gravimetric method 0.2468 gm. of SiO_2 or 29.0 mg. of SiO_2 per gm. of dried tissue.

Sample 4. 0.5 gm. of Liver 407 gave by the colorimetric method 1.33 mg. of SiO_2 per gm. of dried tissue.
50.0 gm. of the same gave by the gravimetric method 0 0660 gm. of SiO_2 or 1.32 mg. of SiO_2 per gm. of dried tissue.

In Isaacs' method the presence of iron in the ash from those tissues containing considerable amounts of blood, such as liver and kidney, resulted in a greenish tint in the test solution. Isaacs diluted his standard with a solution of ferric alum till it matched the green color of the test, and made allowance for the dilution in his calculation. It has been found impossible in this laboratory to get even approximate matches in this way, and the dilution

TABLE I.

Silica Content of Tissues.

		SiO_2 per gm. dried tissue.	SiO_2 per gm. fresh tissue.	Total SiO_2 in organ.
		mg.	mg.	mg.
Lung.	Guinea pig	0.78	0.12	0.50
	" "	0.48	0.06	0.25
	Rabbit	1.70	0.27	2.38
	"	2.02	0.30	2.93
	Dog	2.06	0.32	60.72
	"	2.50	0.39	52.50
	Human (normal)	1.40		
	" (silicotic)	123.55		
Kidney.	Rabbit	1.97	0.25	2.96
	"	1.70	0.34	3.45
	Dog	1.12	0.21	7.28
	"	0.80	0.15	4.88
Liver.	Rabbit	1.90	0.32	10.50
	"	1.85	0.46	9.50
	Dog	1.33	0.34	77.71
	"	0.97	0.23	23.37
Spleen.	Dog	0.66	0.16	1.42
	"	0.49	0.11	0.53

factor in the standard is an additional source of error. In the present method iron is precipitated along with the phosphate, so that the procedure is as applicable to those tissues containing blood, as it is in the case of lung tissue where the iron content is low.

Some results of analyses of animal organs are given in Table I. The values are of the same order as those given by Gonnermann (6) who used the gravimetric method.

Solutions Required.

All reagents must be silica-free, and should be tested for its presence by adding ammonium molybdate and a few drops of sulfuric acid. Of the solutions used, only sodium hydroxide is likely to contain silica. It is therefore necessary to prepare this solution from metallic sodium, when a silica-free reagent will be obtained.

Nitric acid, concentrated.

Ammonium nitrate, 20 per cent solution.

Boric acid, saturated solution.

Normal sodium hydroxide, made by dissolving 2.3 gm. of metallic sodium in water in a nickel crucible, making to 100 cc., and keeping in a paraffined bottle.

Sulfuric acid, 50 per cent by volume.

Magnesia mixture, 5.5 gm. of magnesium chloride and 7 gm. of ammonium chloride dissolved in water and made to 100 cc.

Ammonium Hydroxide 2.5 Normal.—Several samples of aqueous ammonia gave no test for silica. If a positive test is given, a silica-free solution should be prepared by distilling ammonia through a reflux condenser and bubbling the gas into distilled water contained in a paraffined bottle packed in ice. To prevent the water backing up the tube and into the condenser, a slow current of air is sucked, by means of a vacuum pump, through the entire apparatus. Specimens of the solution in the paraffined bottle may be titrated at intervals and the distillation stopped when the desired strength is reached.

Ammonium molybdate, 10 per cent solution.

Standard Picric Acid.—A stock solution is made by dissolving 102.4 mg. of pure vacuum-dried picric acid in water and making to 100 cc. By diluting 1 part of the stock solution to 100 with water a solution is obtained, 50 cc. of which have the same quality and intensity of color as that developed in a solution containing 1 mg. of SiO_2 when treated with 2 cc. of ammonium molybdate and 4 drops of sulfuric acid and made to 50 cc.

Method.

The tissue to be analyzed is dried to constant weight at 105° and finely pulverized in an iron mortar. Ashing is carried out in

the wet way in a platinum crucible of 30 or 40 cc. capacity, though a smaller one may be used. An amount of the tissue (0.2 to 1.0 gm. is a convenient quantity) is treated with 3 cc. of nitric acid, 2 cc. of ammonium nitrate, and 1 cc. of boric acid. The boric acid prevents any loss of silica as fluoride. The mixture is heated on the water bath till frothing ceases and a clear solution is obtained and then to dryness on a hot plate or with a Bunsen flame. Care must be taken during the latter stage, or loss by spattering will occur. The crucible is then heated to redness over a Meker or Bunsen burner. The carbonaceous residue quickly oxidizes, leaving a white ash. Sometimes a second or even a third treatment with nitric acid and ammonium nitrate is necessary; this depends on the amount taken and on the type of tissue. Lung is easily ashed; liver and kidney usually require an additional treatment.

Solution of the silica in the ash is effected by adding 3 cc. of the N NaOH and several cc. of water and heating on the water bath. If the ash sticks to the bottom of the crucible and does not readily dissolve, the process is aided by rubbing it free with a rubber policeman. The crucible is now removed from the water bath, a drop of phenolphthalein added, and then dilute sulfuric acid till the pink color is almost discharged, leaving the solution faintly alkaline. Water is added to a volume of 25 or 35 cc., depending on the size of the crucible being used, and 1 cc. of magnesia mixture added for each 3 mg. of P present. (1 or 2 cc. are usually sufficient except in the case of tissue high in P such as brain.) 2 cc. of ammonia are added, a few minutes allowed for precipitation of the phosphate, and the solution then filtered through a small paper into a Pyrex test-tube graduated at 50 cc. The residue is thoroughly washed with distilled water, the washings being added to the filtrate. After the solution is neutralized, 2 cc. of ammonium molybdate and 4 drops of sulfuric acid are added, and water to 50 cc. The yellow color, which quickly develops, reaches a maximum in 5 minutes and remains constant for several hours.

Test solutions are conveniently matched in Hehner tubes against the picric acid standard, 50 cc. of which have a color equivalent to 1 mg. of SiO_2 in the test. Improvised tubes can be made by sealing a small glass stop-cock in the side near the bottom of one of two 50 cc. graduated cylinders which appear to be identi-

cal as regards kind of glass, bore, height of graduation, *etc.* The standard solution is run out of the tube containing the stop-cock till it matches the test in the other tube. With a little practice, as accurate readings can be obtained with these tubes, when mounted on glass over white cardboard, as are got by the use of an expensive colorimeter. If Nessler tubes are used it is convenient to make several standards of different strength, by appropriate dilution of the stock solution of picric acid.

When the silica content of the tissue is abnormally high, as in silicotic lungs, it is advisable, after the sodium hydroxide is neutralized, to transfer the solution to a graduated cylinder, and use an aliquot portion for the estimation. While the 2 cc. of 10 per cent ammonium molybdate are sufficient for 3.5 mg. of SiO_2,[2] it is desirable that the aliquot portion should contain about 1 mg. since the color of the test should fairly closely approximate that of the standard.

SUMMARY.

A micro method for the estimation of silica in animal tissues, which depends on the production of yellow silicomolybdic acid, has been described. Evidence is produced to show that the method gives accurate results.

The silica content of some tissues has been estimated; values are listed in tabular form.

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[2] Dienert and Wandenbulcke found that 2 cc. of 10 per cent solution of ammonium molybdate were enough to react with not more than 3.5 mg. of SiO_2. This observation has been confirmed by the author.

LACTOSE METABOLISM IN WOMEN.

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HISTORICAL RÉSUMÉ.

Ever since practical methods for the identification of sugar in the urine have found application in medical practice, it has been recognized that there often appears an abnormal amount of sugar, or at least of reducing substances, in the urines of women during confinement.

Lehmann (1), in a text-book published in 1850, reports that Heller, the year before, had observed an increase in the reducing power of urine during lactation. Blot (2) by means of polarimetric and fermentation tests claimed to have established the sugarlike nature of this substance. There followed, during the next 10 years, a controversy, principally between Brücke (3) and Leconte (4) as to the nature of the reducing substance found in the urines of lactating women. It was finally established by

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XUM

Brücke that the substance was a sugar, but it was erroneously assumed by all these early workers that it was the same sugar which occurred in diabetic urines. In 1873, however, de Sinéty (5) observed that these urines did not respond to the fermentation test as they would if glucose were present, and in 1877, Hofmeister (6) isolated from the urine of a lying-in woman a crystalline, optically active substance which was identical in all its properties with lactose. Since the time of this work of Hofmeister's, it has been generally accepted that lactosuria is a common phenomenon among women in confinement. Lemaire (7), in 1895, identified lactose by osazone formation, and quantitatively determined it in the urines of fifteen lactating women. His method was cumbersome and required large amounts of lactose to give a positive test. The same may be said of the method used by Porcher (8–12), who investigated the problem of lactose formation in mammals between the years 1902 and 1910. Porcher's work was mostly done on cows and goats, but in these animals, and also in a number of women, he identified lactose in the urine both before and after delivery. The principal object of his investigation was to prove that the lactose of the milk is formed in the mammary glands and has as its only source the glucose of the blood.

These experiments of Porcher are the latest ones reported in the literature that include observations upon lactosuria in women. What little work has been done on lactose metabolism in more recent years is mainly concerned with the results of injection or ingestion of lactose in normal animals, and without any special consideration of the problem of pregnancy and lactation. The present investigation includes observations on the results of lactose feeding during different stages of the sexual activities of women.

The scarcity of literature upon this subject may be due in part to the fact that there has been no convenient or dependable method of quantitatively analyzing urines for lactose.

Method for Determination of Lactose in Urine.

The following procedure was finally adopted: The urine is analyzed for "total sugar" by the regular Folin-Berglund (13) method; *i.e.*, to a measured amount of urine (from 1 to 10 cc., depending upon its concentration) are added 5 cc. of a 0.1 N solution of H_2SO_4. The volume is then made up to 20 cc. with water, whirled for 2 minutes with 1.5 gm. of Lloyd's reagent, and then centrifuged. With a dry Ostwald pipette, 1 or 2 cc. of the supernatant fluid are transferred to a Folin sugar tube. If only 1 cc. of the solution was used, then 1 cc. of water is added before the 2 cc.

of Folin-Wu copper reagent, and the tube is placed in the boiling water bath. We have boiled for 10 minutes in all determinations and used the 1926 molybdic acid reagent (Folin (14)) for color developer. The reading is made against a 0.2 mg. glucose standard set at 20.0. There are now from 12 to 15 cc. of the solution left in the centrifuge tube, and unless by the foregoing determination it has been found that further dilution is necessary, this solution is transferred directly to an Erlenmeyer flask and put into the water bath to be warmed up to 38–42°. One-quarter of a cake of Fleischmann's yeast is then added, the flask stoppered, and shaken until it is thoroughly emulsified. The flask is then returned to the water bath for exactly 7 minutes, at the end of which time the mixture is immediately transferred to a centrifuge tube, and centrifuged rapidly. Within half a minute the yeast is firmly packed in the bottom of the tube, and we have found this to be the quickest and easiest way to stop the action of the yeast. The turbid supernatant fluid is decanted back into the Erlenmeyer flask, shaken for 2 minutes with 1.5 gm. of Lloyd's reagent, and again centrifuged. The clear supernatant fluid is then ready for the final sugar determination.

In working out the details of this procedure, we made the following observations.

7 minutes is sufficient to destroy glucose in urine up to a concentration of 0.25 per cent. A urine containing more glucose than this must be diluted before fermentation.[1]

A longer fermentation period must be avoided, since it has been found that after the first 10 or 15 minutes the reducing substances of normal urines undergo unexplained fluctuations which make the results on residual reduction unreliable. This finding may explain the fact that conflicting results are often found in the literature wherever fermentation tests have been used upon urine.

Eagle (15), in 1927, reported he had observed that residual reduction values after yeast fermentation were unreliable, and he explained his results on normal urines to which glucose had been added by stating that a "urine-yeast reaction" takes place which

[1] This short fermentation process was worked out in connection with Folin and Svedberg's investigation of the residual reduction of normal blood filtrates, and it is given on p. 418 of that paper, as applied to blood analysis. (Folin, O., and Svedberg, A., *J. Biol. Chem.*, 1926, lxx, 405.)

interferes with the fermentation of glucose. By a preliminary treatment with Lloyd's reagent, he claims that the unknown substances which interfere with the fermentation of glucose are removed. This suggestion of Eagle's was carefully followed up, and it was found that in the short 7 minute fermentation period no such "urine-yeast reaction" takes place, the destruction of added glucose being complete whether the urine is treated with Lloyd's reagent before or after fermentation. However, it was found that if the urine was treated with Lloyd's reagent before fermentation and then cleared of yeast after fermentation by a second 2 minute shaking with Lloyd's reagent, this second treatment caused the further removal of from 0 to 25 mg. per cent of unknown reducing substances. (125 such duplicate analyses were carried out on the urines of pregnant women.) It was ascertained, by the addition of lactose to normal urines, that this further removal of reducing substances by the double Lloyd treatment did not involve any further removal of lactose.

Since the greatest weakness in the fermentation method of analyzing urines for lactose lies in the fact that the non-fermentable reducing substances of the urine include unknown reducing substances as well as lactose, any means of removing these substances without removing lactose is most acceptable. By the inclusion of the double Lloyd treatment in our procedure, this source of error, while by no means eliminated, is at least somewhat decreased.

Somogyi (16) has recently recommended the washing of yeast in his method for the determination of the residual reduction of the blood. We have found that by washing, the yeast blank can be reduced only from 4 or 5 mg. per cent to 1 or 2 mg. per cent. We have also found that in determining the residual reduction of urine by our method a suspension of unwashed yeast gives exactly the same results for residual reduction as does a suspension of washed yeast. But the unexpected observation was made that solid yeast removes from 4 to 8 mg. per cent more reducing substances from urine than does a yeast suspension. Since we have found that this further removal does not include any removal of lactose, it is evident that in the determination of lactose it is better to use solid yeast, which apparently takes out some of the unknown reducing substances as well as glucose.

Of course, if the object were to determine the glucose of the urine, one would use a suspension of washed yeast and avoid the double Lloyd treatment. (When washed yeast is used, no clarification after fermentation is necessary.) It must be borne in mind that, in the method used in this investigation, the difference between the "total sugar" and the "residual reduction" does not represent fermentable sugar alone, but rather includes fermentable sugar plus from 5 to 25 mg. per cent of unknown reducing substances. When the residual reduction is anything over 160 to 200 mg. per cent, we have considered it safe to label the results "lactose." In all analyses, the readings have been made against a glucose standard and the results are expressed in terms of glucose (unless otherwise specified). Glucose is 1.7 times as strong a reducer as lactose (as determined by the Folin-Wu sugar method) and therefore, to get the actual values in terms of lactose, our results must be multiplied by 1.7. Since comparative figures are fully as informative as actual values, and since in many cases we do not feel justified in labelling our results "lactose," we have kept all figures (unless otherwise specified) in terms of glucose.

Before application of this method to the investigation of the problem at hand, it was carefully tested out on a number of urines from normal individuals, diabetics, and lactating mothers. 120 urines were analyzed, and it was found that *except in border-line cases* it was easy to differentiate between glucosuria, lactosuria, and normal urines.

In order to ascertain the rate of lactose excretion the creatinine was also determined. It was found simpler to do this than to attempt to collect 24 hour specimens. Since the daily creatinine output of any individual is constant, and dependent upon the weight (Folin, 1905 (17)), it is possible by knowing the weight of the patient and the creatinine content of a given specimen, to calculate approximately what part of the 24 hour amount 1 cc. of that specimen would represent. Inasmuch as we are interested only in comparative values, however, that is, in following the rise and fall of the lactose excretion, it was decided to express the results in terms of lactose to creatinine ratios: $\frac{\text{mg. lactose per cc.}}{\text{mg. creatinine per cc.}}$ (L:C), "total sugar" to creatinine ratios (TS:C), and residual reduction to creatinine ratios (RR:C). In this way, and by

always using for analysis the morning specimen (before any food has been taken) comparative values are acquired. During the course of this investigation forty-seven analyses were run on the morning urines of normal women and men. The RR:C ratios for the women ran between 0.220 and 0.590, the average being *0.403*. The RR:C ratios for the men ran between 0.250 and 0.520, the average being *0.330*. It was observed that normal individuals vary somewhat in their RR:C level from day to day, the women showing greater fluctuations than the men. This, of course, does not represent sufficient data from which to draw any conclusions concerning the residual reduction of normal urines. It was done simply to give some idea of the normal RR:C level, with which to compare the data on pregnant and lactating women.

The results to be presented, therefore, have been acquired by the use of the method described above. The limitations of this method are fully realized, but it has yielded, nevertheless, considerable data which have proved instructive, and suggest many possibilities concerning the relation of lactose metabolism in women to the processes of reproduction.

Studies on Excretion of Lactose.

If, as seems to have been shown by Folin and Berglund (13), there is no renal threshold for lactose and galactose, then any of these two sugars introduced, either normally (by the glands) or experimentally (by ingestion) into the circulation and not destroyed by the tissues will show up in the urine. It would seem, therefore, that the study of the appearance of lactose (and galactose) in the urines of the individuals experimented upon would give some rather valuable insight into the metabolism of these sugars.

Daily Lactose Excretion of Lactating Women.

The first line of investigation was on the ward patients at the Boston Lying-In Hospital. Since this is an emergency hospital only, the patients do not come in until the day of delivery, and leave from 10 to 14 days after. On twenty-two such women, the daily urines were analyzed. Representative results are given in Table I.

Since it is not practical to present all the data collected, we are

including only a few typical cases. Each individual varies both in the amount of lactose excreted, and in the degree of the fluctuations noted from day to day, but certain generalizations can be made which apply to all the cases observed.

On 90 such specimens a qualitative test, as well as the quantitative determination for total sugar and for lactose, was run. The qualitative test used was the regular Benedict test which is used in most hospitals for the routine urine analysis. We have found that any urine that contains more than 150 mg. per cent of "total

TABLE I.

Daily Urine Analyses on Lactating Women.

Name.....	Mrs. Breslin.		Mrs. R. P.		Mrs. Wagner.	
Remarks....	Normal delivery Feb. 22. More than enough milk.		Normal delivery May 5. Definitely deficient in milk.		Normal delivery Mar. 4. Sufficient milk.	
Days after delivery.	Lactose in urine.		Lactose in urine.		Lactose in urine.	
	mg. per 100 cc.	*L:C*	*mg. per 100 cc.*	*L:C*	*mg. per 100 cc.*	*L:C*
1	38.6	1.28			44.5	1.30
2	61.5	1.23	35	0.442	28.6	1.40
3						
4	310	4.40	63	0.641	1190	8.32
5						
6	500	4.85	133	1.33	981	7.60
7	222	2.89			208	1.36
8	255	2.30	61.8	1.42	Qualitat. Negative.	
9	250	2.50			615	6.48
10	410	3.28				
11	500	6.28			Qualitat. Negative.	
12						
13					Dismissed from hospital.	
14	123	3.39	105	1.12		
15	Dismissed from hospital.					
18			86.5	1.16		
20			86.5	1.16		
23			55	0.810		
26			38	0.550		
30			44.5	0.735		
31			Stopped nursing baby.			

TABLE I—*Concluded.*

Name.....	Mrs. Johnson.		Mrs. Evans.		Mrs. Trudell.	
Remarks....	Normal delivery Mar. 18. Plenty of milk, but discontinued nursing 9th day after delivery.		Normal delivery Apr. 5. Plenty of milk, but discontinued nursing 10th day after delivery.		Still-born baby Feb. 28.	
Days after delivery.	Lactose in urine.		Lactose in urine.		Lactose in urine.	
	mg. per 100 cc.	*L:C*	*mg. per 100 cc.*	*L:C*	*mg. per 100 cc.*	*L:C*
1	148	1.18	54	0.540	23	0.705
2	222	2.12	107	0.910		
3	194	1.61	147	1.17	47	3.76
4	211	1.68	630	7.52		
5	49.4	1.58	235	1.99		
6	510	3.82	335	2.19	16.4	0.740
7	222	1.40	294	1.91	7.7	0.390
8	457	1.83	205	1.24		
9			175	0.890	94	3.10
10	428	2.91	181	0 930	77.5	1.71
11	1000	7.50	176	1.10		
12	333	3.60	157	0.851	66	1.38
13	235	1.15	196	0.835		
14	133	1.33	91	1.18	69	0.660
15	Dismissed from hospital.		103	1.33	Dismissed from hospital.	

* L:C = lactose to creatinine ratio.

sugar" usually gives a positive qualitative test (*i.e.* some yellow precipitate). Normal urines almost never contain more than 100 mg. per cent of reducing substances, but from the figures given in Table I, it is evident that "sugar-positive" urines from women during the first 2 weeks after delivery are frequent. It is not surprising, therefore, that even when qualitative tests alone have been used, it has been a common observation that "sugar" excretion is likely to occur at this time. It will also be evident, however, by comparison of the figures for lactose or residual reduction with those for the L:C ratios, that neither a qualitative nor a quantitative test for "sugar" gives any dependable indication of the actual daily sugar excretion. The time element as represented by the creatinine content of the urine must be determined and taken into account.

The first observation of note is that in every case studied the

L:C ratio on the 1st day after delivery is lower than at any later time. This low level lasts from 1 to 5 days, at the end of which time a sudden and often tremendous rise in lactose excretion occurs. (Mrs. B. C. (Table II) is the most striking example of this that has been observed. Her L:C ratio on the 4th day is 30 times as large as it was just after delivery.) We have not been able to discover that this observation of the low level of the lactose excretion on the first few days after delivery, followed by a sudden very marked excretion of lactose, has been noted before.[2]

The colostrum which is excreted by women during the first few days after delivery differs markedly in both quality and quantity from the milk which usually begins coming on the 3rd or 4th day. Also, during the first few days the new born baby is apparently not getting as much food as it does later for it loses weight. It has therefore been concluded that the mammary glands do not come into full activity until several days after delivery. It was at first thought, therefore, that the amount of lactose appearing in the urine could be taken as an indication of the degree of activity of the mammary glands. Further observations, however, proved that this was not the case. Mrs. Breslin (Table I) had more milk than any other case studied, but her L:C ratios were no higher than those of several other women who had no excess amount of milk. Mrs. B. C. (Table II) showed the highest lactose excretion of any of the cases followed, but her supply of milk was no greater than normal. There were a number of other cases illustrating the point.

Moreover, the great fluctuations in urinary excretion of lactose during the first 2 weeks after delivery, which were evident in all the cases studied, are not explained by any corresponding differences in milk secretion. Attempts were made to find some explanation for these extraordinary fluctuations. The specimens were all taken at the 6 o'clock morning nursing before any food had been taken. It would seem, therefore, that they are comparable with each other. Moreover, it was ascertained that the time of taking

[2] Rowley (18), in following the blood sugar of pregnant and lactating women has been able to discover no abnormalities in blood sugar except sometimes a slight hyperglycemia on the 2nd to the 4th day post partum. This observation is interesting in connection with the above. This worker, however, reports no glycosuria.

the specimen in relation to the time of nursing did not influence the lactose content. Hourly urine specimens showed no change in the L:C ratios before or after nursing. Also the fact that women with still-born babies (three cases were followed) showed these same fluctuations would seem to rule out suckling as an influence upon lactose excretion.

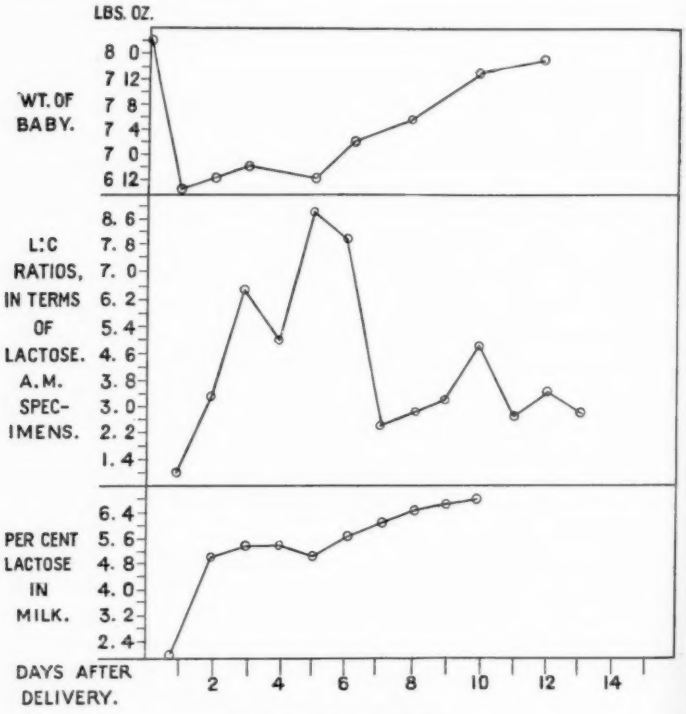

Chart I. Mrs. DiTullio.

Experiments were also carried out in an attempt to discover whether or not the composition of the milk from day to day, or the weight of the babies, had any connection with the fluctuations in L:C ratios. On three women, who were lactating normally, L:C ratios, the weight of the babies, and the lactose content of the

milk were followed daily for the 2 weeks that they were in the hospital. Chart I presents the data on one of the cases. All three of the patients showed practically the same percentage composition and constant rise in lactose content of the milk during the first 2 weeks. Also, all of the babies showed the usual drop in weight just after delivery, and constant increase during the succeeding days. But the fluctuations in the level of the L:C ratios were very marked, and seemed to have no connection with the other two lines of observation.

Two of the cases given in Table I are of women who discontinued nursing. One of the women, Mrs. Johnson, showed a large excretion of lactose on the 2nd day after her breasts were dried up, but with Mrs. Evans, this phenomenon did not occur, although she was having fully as much milk for the baby as was Mrs. Johnson. Porcher (8), in 1902, and Campus (19), in 1920, have observed that, in lactating cows, when suckling is suspended, there appears during the next few days a marked excretion of lactose, the amount of lactose in the urine at this time being in direct proportion to the amount of milk yielded by the cow. It seems quite reasonable that, as Porcher says, there should be a "backing up of lactose" when suckling is discontinued, until the mammary glands cease being active. But with women, at any rate, some individuals seem to be better able to take care of this condition without the help of lactose excretion than others, so that lactose excretion in this condition is not always proportional to the activity of the mammary glands.

On four women who never nursed their babies (Mrs. Trudell, Table I, is an example), the urines were analyzed. Under these conditions the glands in time cease to be active, and the L:C ratios assume a low level, as would be expected. But in all of these cases, up to the time when the glands ceased secreting milk, it was found that the fluctuations in L:C ratios from day to day were much the same as in normal women where suckling was going on.

No parallel can evidently be drawn, therefore, between the activity of the mammary glands and the excretion of lactose. It is, or course, true that where the mother is definitely deficient in supplying milk, the L:C ratios are low (Mrs. R. P., Table I, is the most striking example we have of this). But the reverse, that where the milk supply is large, the L:C ratio will be correspondingly high, evidently does not hold.

The picture presented up to this point was certainly a very complicated one. It was only upon following the urines at later periods during lactation that we were able to obtain any clue to the situation. Table II gives the results with four patients.

All of these women were still nursing their babies at these later dates, and producing more milk than at the earlier periods followed. The fluctuations in lactose excretion had apparently stopped and

TABLE II.

Daily Urine Analyses on Lactating Women.

Mrs. P. B.			Mrs. L. B.		
Normal delivery Dec. 2. Enough milk most of the time.			Normal delivery Dec. 20. Plenty of milk.		
Date.	Lactose in urine.		Date.	Lactose in urine.	
	mg. per 100 cc.	*L:C**		*mg. per 100 cc.*	*L:C*
Dec. 3	47.2	0.842	Dec. 21	48.2	0.862
" 4	152	1 38	" 22	80	1.46
" 5	130	1.97	" 23	365	2.20
" 6	97	1.42			
" 7	86	1.15			
" 8	49.6	0.875			
" 9	84.2	1.20			
" 10	114	1.76			
" 11	110	2.03			
" 12	80	1.56			
" 13	108	1.77			
" 14	97	1.34			
" 15	109	1.68			
" 16	151	1.51			
" 17	116	1.48	Jan. 5	225	2 01
" 18	118	1.30	" 6	180	1.76
" 19	180	1.52	" 7		
" 20	120	1.80	" 8	92	1.45
" 21	135	1.54	" 9		
" 22	123	1.53	" 10	77 2	1 24
Jan. 25	58.8	1.23	Jan. 25	128	1 16
" 26	128	1 35	" 26	62	1.10
" 27	138	1.34	" 27	97	1.12
" 28	198	1.20	" 28	94	1.18
" 29	174	1.18	" 29	153	1.19
" 30	138	1 32	" 30	133	1.13
Feb. 14	17.7	1.12	Feb. 14	55	0.832

TABLE II—*Concluded.*

Mrs. F. B.			Mrs. B. C.		
Normal delivery Dec. 30. Plenty of milk.			Normal delivery Dec. 17. Plenty of milk (no excess).		
Date.	Lactose in urine.		Date.	Lactose in urine.	
	mg. per 100 cc.	*L:C*		*mg. per 100 cc.*	*L:C*
Dec. 31	52.5	0.525	Dec. 18		
Jan. 4	325	5.85	" 19	57.2	0.791
" 5	500	4.00	" 20	106	1.69
" 6	245	2.45	" 21	1390	22.3
" 7	169	2.58	" 22	400	6.66
" 8	160	2.32	" 23	335	4.85
" 9	260	1.82			
" 10	75	2.07			
" 11	250	2.50			
" 12	146	2.90			
Jan. 25	95	1.16	Jan. 27	68.5	1.02
" 26	93	1.19	" 28	34.5	0.940
" 27	112	1.12	" 29	91.0	1.00
" 28	55.2	1.24	" 30	40.5	1.05
" 29	41.7	1.21	" 31	53.5	0.900
" 30	160	1.20	Feb. 1	73.0	0.981
Mar. 9	142	0.855	Feb. 29	8.9	0.902

* L:C = lactose to creatinine ratio.

the L:C ratios were at a constant and lower level. When the urines were tested again at a still later date during lactation, it was found that the L:C ratios were approaching an even lower level, though still somewhat higher than the RR:C values for normal women and men.

It would seem, therefore, that during the first few weeks after the mammary glands come into full activity, some adjustment must be made by the individual. The sudden excretion of lactose by many women on the 2nd to the 4th day might be interpreted to indicate that when milk secretion first starts in, this adjustment has not been made and consequently large amounts of lactose are poured into the circulation and excreted. As the adjustment sets in, the lactose excretion gradually assumes a constant and lower level.

From the data collected, therefore, it would seem safe to assume

that, during the course of lactation, some adjustment is going on which results in a constant decrease in the amount of lactose being excreted, and that the value for the L:C ratios will depend upon the efficiency of this adjustment rather than upon the degree of activity of the mammary glands. This interpretation rationalizes the whole picture. It is not worth while to make any hypothesis as to the nature of this adjustment on the basis of the present data.

Daily Lactose Excretion of Pregnant Women up to Day of Delivery.

On nine women who were in the last weeks of pregnancy, the daily level of the residual reduction of the urine was followed up to the day of delivery. All of these women had normal deliveries. Representative results are given in Table III.

In this particular phase of the investigation we are especially at a loss for any interpretation of the data obtained. In the first

TABLE III.

Daily Urine Analyses on Pregnant Women.

Name.....	Mrs. R. P.			Mrs. Breslin.		
Remarks.....	Normal delivery May 5. Definitely deficient in milk.			Toxemia. Normal delivery Feb. 22. Excess of milk.		
Days before delivery.	Date.	Residual reduction of urine.		Date.	Residual reduction of urine.	
		mg. per 100 cc.	*RR:C**		*mg. per 100 cc.*	*RR:C*
56	Mar. 11	50	0.807			
12				Feb. 10	320	2.01
11				" 11	133	2.06
10	Apr. 25	40.6	0.711	" 12	142	1.20
9	" 26	66.6	0.730	" 13		
8	" 27	94.5	0.705	" 14	364	1.82
7	" 28	59	0.940	" 15		
6	" 29	53.4	0.693	" 16	262	1.83
5	" 30	35.2	0.422	" 17		
4	May 1	52.8	0.550	" 18	364	1.82
3	" 2	41.8	0.710	" 19	80	1.60
2	" 3	63	0.760	" 20	663	2.65
1	" 4	63	0.852	" 21	842	3.79
Delivery.	" 5	108	1.20	" 22	748	4.60
1st day after delivery.	May 6	35	0.442	Feb. 23	38.6	1.28

TABLE III—*Concluded.*

Name.....	Mrs. P. B.			Bernice B.		
Remarks.....	Normal delivery Dec. 2. Plenty of milk.			Still-born baby Mar. 14.		
Days before delivery.	Date.	Residual reduction of urine.		Date.	Residual reduction of urine.	
		mg. per 100 cc.	*RR:C*		*mg. per 100 cc.*	*RR:C*
12	Nov. 20	81.7	0.640			
11	" 21	144	0.789			
10	" 22	141	0.938			
9	" 23	98.2	0.859			
8	" 24	70	0.862			
7	" 25	46.4	0.928			
6	" 26	96.8	1.11	Mar. 8	53	0.640
5	" 27	190	1.03	" 9	183	0.870
4	" 28	81.5	0.903	" 10	40	1.05
3	" 29	196	1.08	" 11	180	1.48
2	" 30	175	1.07	" 12	133	1.52
1	Dec. 1	164	1.29			
Delivery.	" 2	24.5	1.44	" 14	163	2.44
1st day after delivery.	Dec. 3	47.2	0.842	Mar. 15	119	0.650

* RR:C = residual reduction to creatinine ratio.

place, with these urines, most of the values for residual reduction are so low that we do not feel justified in labelling them lactose. On the other hand the RR:C ratios are at a level definitely higher than the normal, and the natural assumption would be that these high values are due to a more or less constant excretion of small amounts of lactose. The strongest support that we have in our own findings for this assumption is the data on the first late pregnancy case studied. This patient, Mrs. Breslin (Table III), a toxemia patient, came to the hospital 3 weeks before delivery, showing a "positive sugar" test in the urine. Analysis of her urines showed that the sugar excreted was non-fermentable, and in large enough amounts (200 to 800 mg. per cent) to justify calling it lactose. It seems logical to consider this an exaggerated case of a common phenomenon, especially since many of the other cases showed at times a residual reduction of the urine amounting to more than 160 mg. per cent. It is difficult to imagine any other non-fermentable reducing substance (that would show up by the

method used) which would be excreted at this time. Also, earlier workers have been able to identify lactose in the urines of pregnant women. Porcher (10), in his observations on women, noted, in several cases, traces of lactose (identified by osazone formation) in the urines of women during the last few days before delivery. His method depended upon the presence of relatively large amounts of lactose. His earlier (8) observations on cows, and Campus' (19) more recent experiments upon these animals may be offered as further proof that, in mammals, lactose is often excreted during the last stages of pregnancy. Both of these workers have found large amounts of lactose in the urines of cows just before labor, and appreciable amounts as much as 16 days before, which is the earliest that analyses were run. The excretion of milk sugar, they report, increases steadily up to the day of calving, at which time it reaches its maximum. Campus has further added that the data on lactose in the urine prior to delivery do not justify a prediction of the subsequent milk production. These data on cows find an extraordinarily close parallel in our findings on women, although in women the lactose excretion is at a much lower level.

On the basis of the foregoing considerations, therefore, it would seem safe to assume that the high level of the RR:C ratios observed in the urines of pregnant women is due to a more or less constant excretion of small amounts of lactose.

With several of these cases the urine was followed for a month or more before delivery, and the RR:C ratios over this period showed a certain amount of fluctuation, but were all decidedly higher than normal values. The most significant change in RR:C level comes during the last days before delivery. In all of the cases studied, the lactose excretion began to rise on the 3rd to 6th day before delivery and reached its maximum on the day when labor set in. On the day after delivery there is a sharp drop to a level which in most cases is lower than that shown at any other time, either before or after delivery. In all but two of the cases studied the lactose excretion on the day of delivery is higher than at any previous times. In these nine cases, the urine analysis was continued for several days after delivery, and it was observed that a slight rise in lactose excretion just before delivery does not necessarily mean that the after delivery lactose excretion will be correspondingly low. Also a very marked rise in the RR:C

ratios during the last days before delivery may be followed by a comparatively low level of lactose excretion during the first 2 weeks of lactation. This fact, that there is no parallel between the lactose excretion before and the lactose excretion after delivery, would seem to show that these two phenomena are not closely connected, and an explanation that would clear up one would probably not apply to the other.

It was also demonstrated by the data collected that findings on lactose output in the urine prior to delivery did not justify a prediction of the subsequent milk production. As would be expected, when the mammary glands are definitely deficient (as in the case of Mrs. R. P., Tables I and III), the RR:C ratios are low both before and after delivery. Also (as in the case of Mrs. Breslin, Tables I and III) a large amount of milk during lactation may be associated with a marked excretion of lactose both before and after delivery. But the fact that the two do not necessarily run parallel was demonstrated by the other cases studied. This would seem to indicate that the phenomenon is not associated with the degree of activity of the mammary glands.

We are at a loss for any interpretation of these prior to delivery data. The after delivery fluctuations in lactose excretion seem to be due to the inability of the system to adjust itself immediately to the large amounts of lactose secreted when the mammary glands come into full activity. On this same basis, the rise in lactose excretion just before delivery might be interpreted to signify an increased activity of the mammary glands at this time. From this point of view, the fall in lactose excretion after delivery would be taken to indicate that the mammary glands cease all activity at this time and then become active again a day or two later. To us, this does not seem an adequate explanation.

Another possibility that suggests itself is that there might be a decrease in lactose tolerance just before delivery. In our later data, however, on tolerance for ingested lactose during pregnancy, we were not able to obtain evidence that the tolerance for lactose does decrease at this time when the RR:C ratio rises. The reason for this rise in lactose excretion just before delivery is therefore entirely obscure. Whether it represents an increase in lactose secretion or a decrease in the ability of the system to take

care of lactose without the help of excretion, it is an entirely unexplained phenomenon.

It is still a mystery to obstetricians what the mechanism is that brings on labor and delivery. Some say that when the fetus reaches a certain size, labor is stimulated. Others have ascribed it to an upset in the endocrine equilibrium at this time. In view of the fact that the whole question is still so much in the dark, the foregoing findings would seem to be of especial interest, while any interpretation of them is impossible.

Lactose Tolerance Tests.

Earlier workers who have experimented upon the urinary excretion following the injection or ingestion of lactose arrived at the conclusion that when lactose or galactose reached the circulation as such they were completely unassimilable. More recent work, especially that of Folin and Berglund (20), has laid this theory open to question. The fact that the tolerance for galactose is increased by the simultaneous ingestion of glucose would seem to show that, in the normal ingestion of lactose, when glucose and galactose supposedly enter the circulation in equivalent amounts, a certain amount of utilization results. Moreover, the enzymatic splitting of lactose into glucose and galactose is limited in the adult intestine, and the ingestion of this carbohydrate is likely to result in the introduction of lactose as such into the circulation. Folin and Berglund (20) have shown that in the male adult the ingestion of 30 gm. of lactose results in the excretion of some unsplit lactose. It is possible to picture that the ingestion of smaller amounts of lactose might result in the introduction of some of this carbohydrate into the circulation as such, and to attribute the failure of it to appear in the urine to an ability of the organism to utilize it in limited amounts.

This is a purely hypothetical suggestion, but if it could be shown that individuals varied in their ability to assimilate lactose, then one would be justified in assuming that the ability to utilize this sugar when introduced into the circulation was probably never entirely lacking. In the course of our work on lactose tolerance tests, it has been observed that, among males, different individuals vary somewhat in their urinary response to ingested lactose, and that, among females, not only is this true, but the same individual

may show a variance in her tolerance toward this carbohydrate at different times. This is offered as support of the suggestion made by Folin and Berglund (20) that the ability to utilize lactose when introduced into the circulation is probably never so entirely lacking as the earlier workers supposed.

However, the main object of these lactose tolerance experiments was not to attempt to prove that lactose was assimilable, but rather to discover what we could about the response of women to the ingestion of lactose, in the hope that it might give some clue to their metabolism of this carbohydrate. It was at first thought that the phenomena in lactose excretion connected with pregnancy and lactation might be attributed to changes in the ability to utilize lactose. Our results on lactose tolerance tests have not shown this to be the case, but they have brought to light certain changes in lactose metabolism which accompany sexual activity in women.

The work of Folin and Berglund (20), in 1922, was the first that was done on lactose tolerance with the use of modern quantitative methods. Practically all of the work which has been done on this subject since that time has been carried out in the attempt to discover the nature of the interrelationship of glucose and galactose in lactose metabolism. The results of these workers, therefore, have no particular bearing upon our own investigation, especially since all of their experiments have been upon male subjects.

Corley (21), 1927, 1928, in his articles on lactose metabolism, decided to work only on male rabbits, for, he states, "the results with female rabbits have not been consistent." This observation is quite suggestive in the light of our own findings on women. Corley himself suggests that the inconsistent results with female rabbits might be associated in some way with differences in galactose utilization at different stages of the sexual cycle or sexual development. The findings which are about to be described show that Corley's hypothesis was undoubtedly correct.

The only recent work on tolerance which has been along the same lines as our own investigation is that of Rowe which has appeared in the literature from time to time during the last 4 years under the general heading of "The Metabolism of Galactose" (22–25). Rowe's work has been entirely on galactose tolerance; that is, the urinary excretion following the ingestion of galactose. He has been particularly interested in the metabolic problems of women, and therefore his work should be of especial interest to us. Unfortunately, however, Rowe's analytical methods are not adequate enough to justify all of the conclusions which he draws. His tolerance test consists of qualitatively testing the urine for "sugar" once before and twice during the 4 hours after the ingestion of 30 or 40 gm. of

galactose. If the urine is qualitatively positive, according to Benedict's test, a quantitative titration is run, but no attempt is made to ascertain the volume or concentration of the urine. Our own work on method has shown that not even a quantitative determination is sufficient in itself to give any indication of the actual sugar excretion. Some method of keeping track of the concentration is necessary. Most workers have done this, in tolerance tests, by collecting the hourly urine specimens and reporting the sugar excretion in mg. per hour. Rowe's omission in using any such method makes his results less valuable than they might otherwise have been. Any tremendous changes in galactose tolerance would probably show up in Rowe's test, and therefore as an aid to the clinical diagnosis of endocrine disorders it may be useful. But the test is not sufficiently instructive to be used as a basis for the conclusions that Rowe draws concerning the metabolism of galactose in normal men and women.

In this investigation the method used in testing the tolerance for lactose has been the following. Whenever possible the subject has gone without breakfast, but we have found that by the 2nd hour after breakfast any glycuresis has disappeared and, therefore, among our volunteers, we have not insisted upon this point. Hourly urine specimens were collected for 2 hours before and for 2 or 3 hours after taking the weighed amount of lactose. The subjects fasted during the course of the experiment. The lactose, dissolved in 100 to 150 cc. of water, was taken immediately after the second urine specimen was passed. In all tests, pure bactolactose was used, a standardized product put out by the Digestive Ferments Company.

In the first tests made, both the excretion of lactose in mg. per hour and the hourly RR:C ratios were determined, and in all cases where we were sure that the total hourly specimens were carefully collected these two forms of expressing the results checked. But in many of the subjects we could not be sure that the exact hour of collecting the specimen would be kept track of, or that the total specimen was always saved. It was therefore decided to keep all results in terms of RR:C ratios. It is perfectly evident, from the data collected, that the rise in RR:C level is a response to the ingested lactose. We have not attempted to differentiate between lactose and galactose excretion, since this phase of the problem does not particularly concern this investigation. Since most of the doses of lactose were less than 30 gm., however, we assume, on the basis of Folin and Berglund's (20) results, that the rise in RR:C level represents galactose

excretion. Our particular concern is the utilization of lactose, and if the ingestion of a given amount does not result in urinary excretion of either lactose or galactose, it seems safe to assume that this sugar has been assimilated.

Lactose Tolerance of Normal Male Adults.

For the purposes of comparison, tests were first run on nine normal men. Folin and Berglund (20) report 10 gm. of lactose as the tolerance dose for the normal male adult. Our own data check with this finding. Table IV represents a typical case.

TABLE IV.

Urinary Response of a Normal Male to Ingestion of Lactose.

Subject P.L.

Time.	Remarks.	Experiment 1. 20 gm. lactose.		Experiment 2. 10 gm. lactose.		Experiment 3. 5 gm. lactose.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		mg. per 100 cc.	*RR:C**	*mg. per 100 cc.*	*RR:C*	*mg. per 100 cc.*	*RR:C*
	No breakfast.						
9.00 a.m.	Fasting.	80	0.391	56.8	0.410	80	0.520
10.00 "	Took lactose.	40	0.360	76	0.360	54	0.415
11.00 "	Fasting.	48.5	1.01	40	0.600	57.2	0.388
12.00 noon.	"	8	0.436	12.5	0.448	78.5	0.415
1.00 p.m.	"	52.2	0.410	53.5	0.322	84	0.420

* RR:C = residual reduction to creatinine ratio.

The results of Folin and Berglund are in terms of total sugar excretion in mg. per hour. In all of our tolerance tests we have analyzed for total sugar as well as residual reduction, and although the two do not always run exactly parallel, in general the same results would have been acquired for tolerance dose whether the urine analyses were reported in terms of TS:C or RR:C ratios. However, the urinary response to the ingestion of lactose is usually more decidedly evident when the results are expressed in terms of residual reduction rather than total sugar.

The fact that the residual reduction in mg. per 100 cc. gives absolutely no indication of the actual level of the excretion is more

than evident. We have included these figures in the tables, however, since, in connection with the RR:C ratios, they give an indication of the concentration of the urine specimens, and show whether or not diuresis followed the ingestion of lactose. The fact that, in the case of P. L. (Table IV), diuresis occurred in the two experiments where the urinary response to the ingestion of lactose was evident, is not to be taken as significant, for the results on other individuals show that this is by no means the rule.

Although 10 gm. may be considered the tolerance dose for the normal male, we have found that all men do not respond to the ingestion of this amount of lactose with the same degree of rise in the RR:C level of the urine. The response of one of the subjects was barely determinable, the rise of the RR:C level being only from 0.270 to 0.298. Another showed a rise in RR:C level from 0.335 to 0.695 after the ingestion of 10 gm. of lactose. The others ranged between these two extremes. In practically every case the RR:C level was back to normal by the end of the 2nd hour after ingestion of the lactose.

These tests on men were carried out for the purposes of comparison and will be referred to again in connection with the results of like tests on women.

Lactose Tolerance of Normal Women at Different Stages of the Sexual Cycle.

It was not at first expected that any difference would be found between the response of normal men and that of normal women to the ingestion of lactose. It was therefore a surprise to discover, in the case of the first test tried on a normal woman, O. W., that the individual showed no urinary response to the ingestion of 20 gm. of lactose. No men have been observed who did not respond to 10 gm. However, when it was attempted at a later date to repeat this observation on O. W., it was discovered that the individual not only responded to 20 gm., but showed as great a response to 10 gm. as did any of the normal men. It naturally occurred to us at this point that the tolerance for lactose in women might be influenced by the sexual cycle, and our results on the seven women studied showed that this is the case. These seven women varied in age from 23 to 33 years, and all of them are unmarried.

TABLE V.

Urinary Response of Normal Women to Ingestion of Lactose.

Subject O. W.; response to ingestion of 20 gm. lactose.

Time.	Remarks.	Intermenstrual period. 11th day of sexual cycle.		2nd day before menstruation. 28th day of sexual cycle.		2nd day of menstruation.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		mg. per 100 cc.	*RR:C**	*mg. per 100 cc.*	*RR:C*	*mg. per 100 cc.*	*RR:C*
7.30–10.30 a.m.	Breakfast at 8.00 a.m. Fasting.	70	0.557	15.4	0.723	38.2	0.395
11.30 a.m.	Took lactose.	33.5	0.520	16.7	0.556	36.5	0.393
12.30 p.m.	Fasting.	12.3	0.950	53.3	0.374	10.2	0.432
1.30 "	"	33.7	0.500			34.2	0.425
2.30 "	"	13.4	0.500	28.2	0.425	22.6	0.405

Subject J. D.; response to ingestion of 10 gm. lactose.

Time.	Remarks.	Intermenstrual period. 8th day of sexual cycle.		2nd day before menstruation. 26th day of sexual cycle.		1st day of menstruation.	
8.30–9.30 a.m.	Breakfast at 7.30 a.m.			25.8	0.602		
10.30 a.m.	Fasting.	80	0.480			32.5	0.440
11.30 "	Took lactose.	69.5	0.450	79	0.685	37.5	0.400
12.30 p.m.	Fasting.	55	0.550	84	0.650	36.2	0.415
1.30 "	"	61.5	0.382	94	0.640	61.2	0.350

Subject M. E.; response to ingestion of 10 gm. lactose.

Time.	Remarks.	Intermenstrual period. 11th day of sexual cycle.		2nd day before menstruation. 26th day of sexual cycle.		2nd day of menstruation.	
8.00–10.00 a.m.	Breakfast at 8.00 a.m. Fasting.	51.5	0.325	153	0.602	45.5	0.455
11.00 a.m.	Took lactose.	59	0.322	80	0.677	33	0.420
12.00 noon.	Fasting.	20	0.445	32.7	0.570	22.2	0.400
1.00 p.m.	"	13.5	0.292	12.1	0.660	18.2	0.406

* RR:C = residual reduction to creatinine ratio.

At first no special plan was followed as to the time of making the tests, since we had no idea what to expect. But it was soon evident that it was just before and during menstruation that the effect on lactose tolerance was marked. Ovulation does not seem to be concerned with this phenomenon. The course followed therefore was to find out what the tolerance was in the intermenstrual period, and then to test out the response to this amount of lactose once just before and once just after the beginning of menstruation. Representative results are given in Table V.

TABLE VI.

Urinary Response to the Ingestion of Lactose of a Woman Who Apparently Is Not Normal.

Subject E.P.; response to ingestion of 10 gm. of lactose.

Time.	Remarks.	Intermenstrual period. 12th day of sexual cycle.		4th day before menstruation. 26th day of sexual cycle.		3rd day of menstruation.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		mg. per 100 cc.	*RR:C**	*mg. per 100 cc.*	*RR:C*	*mg. per 100 cc.*	*RR:C*
7.30–9.30 a.m.	Breakfast at 7.30 a.m.	88	0.486			66	0.365
10.30 a.m.	Fasting.	92	0.498	63	0.380	75	0.355
11.30 "	Took lactose.	55.3	0.499	59.9	0.387	58	0.385
12.30 p.m.	Fasting.	407	3.00	286	2.46	305	2.15
1.30 "	"	139	0.940	128	0.605	96.2	0.582

* RR:C = residual reduction to creatinine ratio.

Only two subjects were found who did not show, in the intermenstrual period, a response to 10 gm. of ingested lactose, the rest of the normal women showing, at this time, a urinary response which was entirely comparable to the response of normal men. The effect of the sexual cycle upon the lactose tolerance was evident in every case studied. In most cases the effect is most marked just before menstruation, and, in all but one of the cases studied, the tolerance has been found to be increased at this time to such an extent that there is no response to the ingestion of 10 gm. of lactose, and in some cases no response to the ingestion of

20 gm. During menstruation also, it has been found that the tolerance is greater than during the intermenstrual period.

In the course of our work upon the lactose tolerance of normal women we have happened upon one individual whose tolerance is decidedly abnormal. Table VI gives the results of three of the tests made upon her.

The data show that even this abnormal subject is affected in her lactose tolerance during menstruation, the rise in RR:C ratio being less at this time than in the intermenstrual period. As far as E.P. knows, she has no physical abnormalities. She shows no signs of glycosuria, nor is her glycuresis after meals any more marked than that of the normal individual,—except, of course, when the meal contains any amount of milk. The only illness in her history is a very serious nervous breakdown 3 years ago, from which she has entirely recovered. In the light of Rowe's work on galactose tolerance (24), it is entirely possible that E.P.'s abnormally low tolerance for lactose is due to some endocrine disorder. According to Rowe, increased function of the posterior lobe of the pituitary, ovarian failure, or adrenal failure may cause a lowering of the tolerance for galactose.

From the data on E.P., it is seen that her abnormal response to lactose ingestion would be evident even if only a qualitative test of the reducing power of the urine were used, since anything over 150 mg. per 100 cc. gives a positive test with Benedict's reagent, the one used by Rowe in his experiments. It is equally evident, in looking over the residual reduction in mg. per 100 cc. in Table V, that Rowe's method of making the tolerance test would be inadequate as an indication of the changes in tolerance of normal women. Rowe reports that the tolerance dose of normal women is in general 10 gm. higher than that of normal men, and that during menstruation the tendency is for the tolerance to *decrease*. He places the normal tolerance dose at 30 gm. for men and 40 gm. for women. It is not surprising that Rowe's results do not agree with our own when one considers the difference in methods. The fact that Rowe feeds galactose while we are feeding lactose would probably not explain the differences in results, since it has been found (Folin and Berglund) that the tolerance for galactose is even lower than that for lactose. In lactose feeding it is probable that the principal sugars that reach the circulation are galactose and glu-

cose. It is therefore safe to assume that any changes in tolerance observed in women during sexual activity would be in the same direction whichever sugar were fed.

We do not attempt to offer any explanation why the lactose tolerance of normal women should increase at the time of menstruation. Rowe interprets his findings of higher tolerance for galactose in women than in men as indicating that the mammary glands are able to absorb this sugar. It might, of course, be possible that at the time of menstruation some mammary activity went on which caused the absorption of galactose. It seems, however, to have been pretty well proved that the mammary glands use only glucose in the secretion of milk (Porcher (9)), and there is therefore no real reason to suppose that they would absorb galactose at any time. Rowe's own findings of the profound effect of endocrine abnormalities upon galactose tolerance suggest a more likely source of explanation, especially since the endocrine glands are so clearly associated with sexual phenomena. However, we merely present the facts which our observations have made clear; namely, that in most normal women the lactose tolerance is the same as that of normal men, except just before and during menstruation, at which time it is increased.

Lactose Tolerance of Pregnant Women.

The finding of an increased tolerance for lactose in women at the time of menstruation led us to expect that pregnancy would surely have some effect upon the urinary response to the ingestion of this carbohydrate. It was not surprising, therefore, to find that a marked increase in the tolerance for lactose is coincident with pregnancy. Among the seventeen pregnancy cases followed there was only one that showed any urinary response to the ingestion of less than 20 gm. of lactose. In a number of the cases the response to 20 gm. was very slight (less than the normal response to 10 gm.), and with five of the pregnant women 30 gm. were found to be the tolerance dose.

For this part of the investigation we were extremely fortunate in having at our disposal, through the kindness of Dr. Foster Kellogg, the Florence Crittenton Home Hospital, where normal pregnancy cases are accommodated for as many as 5 months before delivery and 2 months after. All of these subjects went without breakfast on the days that the test

was tried, and, thanks to the very intelligent cooperation of the nurse in charge, we could always be certain that the tests would be carefully carried out.

TABLE VII.

Urinary Response of Pregnant Women to Ingestion of Lactose.

Mrs. Noring. Normal delivery Mar. 1. Milk sufficient.

Time.	Remarks.	10 gm. lactose Feb. 21.		20 gm. lactose Feb. 23.		30 gm. lactose Feb. 24.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		mg. per 100 cc.	*RR:C**	*mg. per 100 cc.*	*RR:C*	*mg. per 100 cc.*	*RR:C*
6.00 a.m.	Fasting.	48.5	0.760	53.4	0.960	49	0.620
7.00 "	Took lactose.	15.8	0.789	76.1	0.886	45	0.605
8.00 "	Fasting.	51.5	0.762	73	0.880	49	0.830
9.00 "	"	44	0.750	76.1	0.882	48.5	0.720
10.00 "	"	26.8	0.750	66.2	0.860	48.5	0.655

Lillian. Normal delivery May 11. Milk sufficient.

Time.	Remarks.	20 gm. lactose Mar. 23.		10 gm. lactose Mar. 28.		20 gm. lactose May 9.	
6.45–7.45 a.m.	Fasting.	30	0.605	33.3	0.880	44	0.628
8.45 a.m.	Took lactose.	34.5	0.590	36.5	0.840	31.7	0.606
9.45 "	Fasting.	91	0.800	26.6	0.820	76.2	0.671
10.45 "	"	108	0.700	114	0.850	71.1	0.690
11.45 "	"	100	0.580	118	0.855	69	0.621

Edith I. Due to be delivered on Aug. 15.

Time.	Remarks.	20 gm. lactose Mar. 29.		10 gm. lactose Mar. 30.		20 gm. lactose May 10.	
6.45–7.45 a.m.	Fasting.	24	0.630	12.6	0.765	113	0.705
8.45 a.m.	Took lactose.	20	0.538	89	0.750	46.2	0.694
9.45 "	Fasting.	40	0.535	114	0.745	68.4	0.718
10.45 "	"	69	0.670	160	0.740	95.2	0.876
11.45 "	"	50.8	0.735	131	0.755	114	0.886

* RR:C = residual reduction to creatinine ratio.

The procedure followed on pregnancy cases was first to try the test with 20 gm. of lactose; if there was no urinary response to repeat the test with 30 gm. If, on the other hand, there was a

response to 20 gm., 10 gm. were tried in order to make sure that 20 gm. were the tolerance dose. In the cases of early pregnancy, the test was repeated at a date nearer to delivery to see if the tolerance changed as pregnancy advanced. Representative results are given in Table VII.

The most noticeable difference in lactose excretion between normal and pregnant women, aside from the increased tolerance of the latter, is in the delayed rise in the curve for the residual reduction of hourly urine specimens after lactose ingestion. In normal women, as in normal men, the maximum rise in RR:C level comes during the 1st hour after the ingestion of lactose, and in almost every case, with normal men, and with women whether menstruating or not, the RR:C level is back to normal by the end of the 2nd hour after the lactose was taken. This is quite evidently not true with pregnant women. In many of the cases studied the maximum response is not until the 2nd or 3rd hour after ingestion, and the return to normal is likewise slower than in the normal individual.

It has been quite evident from the results on the cases tested at the end of pregnancy, which we have been able to observe after delivery, that the amount of increase in tolerance during pregnancy is in no way related to the amount of milk that the women have for their babies. It almost seems, therefore, that here again the explanation for the phenomena observed would not be found to have anything to do with the degree of activity of the mammary glands.

As far as we could discover, there is no relation between the tolerance for lactose and the daily RR:C ratios during pregnancy. In several cases the tolerance test was made a day or two before delivery when the RR:C level had begun to rise, but in general it was found that at this time the tolerance for lactose was as high or higher than it had been earlier, when the RR:C ratios were lower. The increase in lactose excretion which has been observed to occur just before delivery cannot, therefore, be attributed to a decrease in lactose tolerance.

In some of the cases followed it appeared that the lactose tolerance increased as pregnancy advanced, and that the tendency was for the urinary response after the ingestion of lactose to be slower in manifesting itself as the time for delivery approached.

However, it was evident from our data that no generalization can be made upon this point.

As in the case of the effect of menstruation, here again our results do not agree with those of Rowe. Rowe finds that the tolerance of pregnant women for galactose, especially in the later stages, is markedly lowered. An examination of our own data on this part of the investigation made it quite evident how easy it would be to draw incorrect conclusions concerning the tolerance of pregnant women if one used a method of testing the urine which did not take into account the concentration of the specimen. In the first place the reducing power of the urines of pregnant women is in general higher than that of normal women as we have already shown, so that a smaller rise in reducing power would push it above the value where it would give a qualitative test for sugar. In the second place, as is seen from the cases given in Table VII), with pregnant women, the urines after the lactose is taken are in many cases more concentrated than before. This would make it possible to get a qualitatively positive test when actually the rate of sugar excretion had not changed. The tendency toward dehydration after the ingestion of lactose among pregnant women is, in itself, worthy of note. Among normal women and men, diuresis is more likely to occur.

Rowe ascribed the increased tolerance of normal women, which he observed, to a capacity for storage of galactose in the mammary glands. His interpretation of his finding of a "lowered tolerance during pregnancy" is that the mammary glands have started their synthetic activity and have gradually become saturated so that their storage capacity is practically eliminated. We shall make no attempt to interpret our findings on the changes in lactose tolerance coincident with pregnancy. However, we are inclined not to ascribe these changes to mammary activity, especially in the light of the results obtained from tolerance tests on lactating women.

Lactose Tolerance of Lactating Women.

The uniformity of the results in this part of the investigation was rather surprising, and left no question as to the urinary response of lactating women to the ingestion of lactose. In all of the ten cases experimented upon, 10 gm. of lactose were found to be

the tolerance dose. Also the type of curve for the hourly RR:C ratios was the same as that found in normal women and men, the maximum response coming the 1st hour after ingestion, and the return to normal being complete, in most cases, by the end of the 2nd hour after the lactose was taken. Representative results are given in Table VIII.

With six of the cases the tolerance dose before delivery had been determined. Four of these had shown, during pregnancy, a tolerance dose of 20 gm., one of 30, and the other of 10 gm. of lactose. The tolerance of lactating women seems to be in no way

TABLE VIII.

Urinary Response of Lactating Women to Ingestion of Lactose.

Time.	Remarks.	Mrs. P. B. Normal delivery Dec. 2. Enough milk most of time. 10 gm. lactose Feb. 14. Residual reduction of urine.		Bernadetti. Normal delivery Mar. 20. More than enough milk. 10 gm. lactose Apr. 10. Residual reduction of urine.		Rebecca B. Normal delivery Apr. 13. Milk often insufficient. 10 gm. lactose May 16. Residual reduction of urine.	
		mg. per 100 cc.	*RR:C**	*mg. per 100 cc.*	*RR:C*	*mg. per 100 cc.*	*RR:C*
6.45–7.45 a.m.	Fasting.	89	1.08	193	1.56	28	0.460
8.45 a.m.	Took lactose.	59.3	1.01	177	1.59	40	0.410
9.45 "	Fasting.	50	1.20	145	1.72	48.2	0.590
10.45 "	"	17.7	1.12	156	1.56	52.6	0.472

* RR:C = residual reduction to creatinine ratio.

connected with what the tolerance of the individual was before delivery, nor with the amount of milk for the baby, nor with the daily level of the RR:C ratios either before or after delivery. In all cases 10 gm. of lactose are the smallest amount which will cause a rise in the RR:C ratio of the urine.

Rowe examined ten women during the first 2 weeks after delivery and found their tolerance very low. We have not attempted to test women during this period since we considered that the high lactose excretion which is evident at this time, and especially the great fluctuations in R:C ratios, made any tolerance test in which the changes in level are so small, entirely unpracticable. For

these same reasons we are inclined to question Rowe's results on this point, and to ascribe his finding of a low tolerance to the fact that the reducing power of the urines of lactating women at this time is usually very high. Our earliest test on a lactating woman was made 3 weeks after delivery, and the latest test was 2½ months after delivery. We are inclined to believe that immediately after delivery the lactose tolerance of women returns to the normal 10 gm. level shown by normal men, and by most normal women in the intermenstrual period.

It would seem, therefore, that after delivery, during the months of lactation, whatever influences cause the increase in tolerance for lactose at the time of menstruation and during pregnancy have ceased to exert themselves. The mammary glands, during lactation, are at the height of their activity, and the fact that the tolerance is unchanged would appear to furnish rather strong support of the previous hypothesis, that the phenomena observed in the course of this investigation probably cannot be considered as due to the degree of activity of the mammary glands.

DISCUSSION AND RÉSUMÉ.

This investigation is principally concerned with the presentation of data on lactose excretion in women. Little in the line of interpretation is possible, but the accumulation of accurate results is necessary for future work on the subject, and helps eliminate the possibility of drawing incorrect conclusions.

It has been found that during pregnancy and lactation, lactose is found in the urines of women in varying amounts. During the first few weeks after delivery lactose is often excreted in very large amounts (as high as 4 per cent) and the fluctuations in excretion from day to day are marked. There is evidence that this excretion starts in when the mammary glands first assume full activity and that it is due to a lack of some sort of adjustment which later becomes evident and causes the residual reduction to creatinine (RR:C) ratios of the urine to assume a constant and lower level. It has been found that the tolerance for ingested lactose at this time is the same as in normal individuals, and therefore the gradual decrease in lactose excretion as lactation advances cannot be considered as due to an increase in lactose tolerance. It is possible that the adjustment is in the mammary

glands themselves. It seems reasonable that in the course of lactation, the mammary glands should adjust themselves to produce exactly as much lactose as is needed for the milk and no more, so that there would be little or no escape of excess lactose into the circulation. The fact that during the first 2 or 3 weeks the adjustment is so far from complete, and that even at the end of 3 months there is still some excretion of lactose is not, however, in keeping with the usual efficiency of the animal machine.

The appearance of lactose in the urine during pregnancy cannot be ascribed to a decrease in the tolerance for this sugar for tolerance tests show that in pregnancy the tolerance for lactose is markedly increased. It is possible, however, that owing to a certain amount of mammary activity at this time, lactose is present, and, although the tolerance is high, showing that some assimilation is possible, it is not so high but that a little lactose escapes into the blood stream and appears in the urine.

A striking increase in lactose excretion just before delivery has been observed. It has been shown that this phenomenon is not related to the degree of activity of the mammary glands, nor to the lactose excretion after delivery. Also, the experimental evidence seems to show that the lactose tolerance does not decrease at this time. Whatever the explanation of this increased excretion may be, it seems quite possible that it is caused by the same metabolic change which brings on labor and delivery. This latter phenomenon is at present entirely unexplained, and for this reason the foregoing observation is particularly suggestive.

The results on lactose tolerance tests have brought to light many peculiarities in the lactose metabolism of women, although they do not appear to furnish any explanation of the observations on the daily level of lactose excretion during pregnancy and lactation. These tolerance tests do, however, provide further evidence of the fact that there is a decided upset in the metabolism of lactose associated with female sex activity.

The natural assumption would be that all the phenomena observed must be associated with the mammary gland, since this organ is concerned with the secretion of lactose. It was to the mammary glands that Rowe turned in the interpretation of his findings. From the data which we have been able to gather, however, we are inclined to doubt whether it is this gland which is

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involved. None of the phenomena observed seem to be related in any way to the degree of activity of the mammary glands. Rowe (24) has found that endocrine abnormalities have profound effects upon the tolerance for galactose. Considering the fact that the endocrine glands are so closely associated with sexual activity, it seems entirely possible that the interpretation of all of the data that have been collected in the course of this investigation may lie within the field of endocrinology.

The observations that have been made, therefore, upon the urinary excretion of lactose in normal, pregnant, and lactating women demonstrate that certain peculiarities in lactose metabolism are associated with the sexual activities of women. In conclusion, a brief outline is presented of the experimental data which have been collected.

1. During the last stages of pregnancy there is a more or less constant excretion of small amounts of lactose.

2. During the last few days before delivery there is a sudden very marked rise in lactose excretion, which reaches its height on the day of delivery.

3. After delivery, the lactose excretion immediately drops to a low level where it remains for from 2 to 5 days. There is then a sudden and often tremendous excretion of lactose and, during the first few weeks of lactation, the fluctuations in the excretion of lactose are very marked.

4. By the end of the 1st month after delivery the lactose excretion has assumed a constant and lower level, and this level slowly approaches the normal values for residual reduction of the urine as lactation progresses.

5. The tolerance for lactose of most normal women is, in the intermenstrual period, the same as that of normal men, the tolerance dose being 10 gm.

6. Menstruation causes an increase in the tolerance of women for lactose, so that at this time many women show no urinary response to the ingestion of 20 gm. of lactose.

7. During pregnancy the tolerance for lactose is increased, being in some women 3 times as high as in other individuals. Also the urinary response to the ingestion of lactose is slower in pregnant women, and the return to normal is correspondingly slow.

8. During lactation the tolerance for lactose is apparently the

same in every way as it is in normal men and in most women in the intermenstrual period.

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SOME FACTORS AFFECTING THE ADSORPTION OF QUININE, OXALATE, AND GLUCOSE BY FULLERS' EARTH AND NORIT.*

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The purpose of this investigation was to make a study of some of the factors affecting the adsorption of electropositive, electronegative, and non-electrolytic substances of known composition, by different adsorbents. Such a study should furnish a basis for a more efficient utilization of these adsorbents in the fractionation of the vitamin B complex. Since fullers' earth and norit had been used in this laboratory and elsewhere (1–5) for the adsorption of at least two factors of the vitamin B complex, the studies have been restricted to these adsorbents.

The available literature contains numerous references concerning the adsorption of various substances by these adsorbents, and the results have been published in some excellent summaries. Mention will be made here only of those references which relate directly to this investigation. The fact that fullers' earth carries an unusually large proportion of colloidal material of high adsorptive capacity was pointed out by Parsons (6). The ability of fullers' earth to adsorb alkaloids was probably first noted by Lloyd (7) in 1910. This investigator observed that different earths had different affinities for a given alkaloid. He attempted to isolate the active constituent from the earth, but such an attempt was unsuccessful. He found, however, that the activity could be destroyed by heating the earth to a red heat, and therefore, concluded that the alkaloidal affinity of the adsorbent depended upon its physical condition, which in turn rested upon and was closely connected with the hydrated condition of the material. Increase in hydration resulted in an increase in this affinity. Gordin and Kaplan (8) found fullers' earth to be more specific for alkaloids than other adsorbents. These investigators found that both acids and alkalies were

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adsorbed by fullers' earth and charcoal, but the presence of free acids did not affect the adsorption of alkaloids. Aluminum hydroxide was found ineffective as an adsorbent for alkaloids, glucosides, and bitter principles.

Haber (9) states that in crystals, the molecules are held together by the forces of valences, and in general these valences are satisfied; the molecules on the surface, however, are not completely saturated, and the residual valence on the surface represents the field of possible adsorption.

According to Freundlich (10) adsorption by a solid adsorbent proceeds parallel with surface energy. Michaelis and Rona (11), however, fail to agree with this general conception of adsorption. These investigators hold that there is no surface-active non-electrolyte which is adsorbed in the slightest degree by either kaolin or ferric hydroxide. Schulze (12) states that in the process of adsorption by many amorphous insoluble silicates, the total basic content of the adsorbent is replaced by bases from the solution. This view is substantiated by the findings of Gottwald (13), who combined solutions of sodium silicate and aluminum sulfate, and found that the resultant gelatinous precipitate was composed primarily of aluminum silicate contaminated with a small amount of sodium sulfate. Further light was thrown upon this type of adsorption by the investigations of Rona and Michaelis (14) who found that electroequivalent quantities of ions are given off and adsorbed during the process of adsorption. The investigations of Marc (15) show the rate at which this type of adsorption takes place and suggest that the phenomena are similar in this respect to many ordinary reactions.

The adsorption of substances by carbons appears to present even more complex phenomena. Bartell and Miller (16) believe that the reaction accompanying adsorption by carbons is hydrolytic in nature and that under certain conditions acids may be adsorbed, while under different conditions bases may be adsorbed. Freundlich and Losev (17) found that when a solution of new fuchsin in the form of its hydrochloric acid salt was treated with charcoal, the outside liquid became colorless but retained all the chlorine ions. Kolthoff (18) made a study of this type of adsorption using an ash-free sugar coal prepared according to the methods of Bartell and Miller (16) and found that this substance adsorbed electrolytes only slightly. What little adsorption did take place was attributed to the small quantities of impurities which failed to be removed, and which offered some chance for ionic exchange.

Tanner (19) brought out the similarity between the process of dyeing and the adsorption of coloring matter by carbons. According to this author, the relationship of the charge of the adsorbent to the charge of the substance adsorbed is possibly the most important factor in the dyeing process as well as in adsorption by carbons. Traube (20) found that many substances, for example organic acids, alcohols, and amines, greatly lowered the surface tension and therefore the surface energy of their aqueous solutions, which created a most favorable condition for adsorption, especially by carbons.

Although the general effect of acid and alkali upon the adsorption of

coloring matter from solutions by carbons has been known for some years, no systematic study of these factors was made until Brewster and Raines (21) studied the effect of H ion on the decolorization of cane juice. These investigators found that decolorization increased with increase in H ion concentration and advocated the adsorption of these substances at pH 4.0. More recently Blowski and Bon (22) reported similar effects upon raw cane sugar liquids.

Hauge and Williaman (23) have recently published the results of an extensive investigation of the effect of H ion concentration on the adsorption of electropositive, electronegative, amphoteric, and non-electrolytic substance by carbons. These investigators concluded that carbons adsorbed the greatest quantity of electronegative substances from the most acidic solutions, while alkalinity favored the adsorption of electropositive substances; amphoteric substances were adsorbed more strongly at their isoelectric points, while the adsorption of a non-electrolyte by carbons was unaffected by the H ion concentration of the solutions.

With the results from the investigations mentioned above in mind, we undertook to determine the effect of certain factors upon the adsorption of a cation, an anion, and a non-electrolyte by fullers' earth and norit. The major portion of our investigation has been restricted to fullers' earth as the adsorbent since this substance had previously been used rather extensively in this laboratory. Quinine, oxalate, and glucose were selected as representatives of the three different types of substances to be studied. The investigation has been confined chiefly to such factors as H ion concentration, heating the adsorbent previous to adsorption, fineness of particle, electrodialysis of adsorbent, and the extraction of the adsorbent with hot acid solution.

Adsorption of Quinine.

Experimental Procedure.

A series of aqueous solutions was prepared by dissolving 1 gm. of quinine bisulfate in 500 ml. of distilled water, adding the theoretical quantity of H_2SO_4 or NaOH to make approximately the desired pH, and then diluting to 1000 ml. with distilled water. All solutions which contained any undissolved quinine or showed any degree of turbidity, other than the fluorescence characteristic of this alkaloid in acid solution, were discarded. After the solution had attained equilibrium, the H ion concentration was determined by means of the hydrogen electrode. In this manner,

a series of ten solutions ranging in pH from 0.24 to 8.29, and containing 1 mg. of quinine bisulfate per ml., was prepared. It was not possible to cover the desired range of pH values owing to the insolubility of quinine in aqueous solutions more alkaline than about pH 8.29. There was prepared, therefore, another series of quinine solutions similar to the first except that aqueous alcohol (63.3 per cent by weight of ethyl alcohol)was used as the solvent. This series consisted of thirteen solutions ranging from pH 1.30 to pH 14.34 and containing 1 mg. of quinine bisulfate per ml.

After numerous preliminary tests, the following procedure was adopted for measuring the adsorptive capacity of the various adsorbents for quinine bisulfate: 0.2 gm. portions of the finely ground (150 mesh) fullers' earth were weighed accurately and placed in a number of dry 100 ml. Erlenmeyer flasks. Separate 50 ml. portions of the quinine solutions were added to each of the flasks by means of a pipette, care being taken to use a clean dry pipette for each portion. The flasks were shaken by hand at 5 minute intervals, for 25 minutes; each solution was then filtered through a small quantitative filter into a 50 ml. volumetric flask. About 20 ml. of each filtrate were used for pH measurement. Duplicate portions of 10 ml. of each filtrate were pipetted into small beakers, diluted with 10 ml. of distilled water, and made strongly acid with 1:1 sulfuric acid. The cold solutions were then titrated with $KMnO_4$. Blank determinations were made to determine the amounts of quinine adsorbed by the filter paper at the various H ion concentrations, also to ascertain the amount of reducing substances removed from the various fullers' earths by aqueous solutions of the same pH as the quinine solution. The data presented have been corrected whenever these errors became apparent.

The adsorption from the alcoholic solutions was carried out in a manner similar to that of the aqueous solutions. The presence of alcohol was found to complicate the permanganate titration; consequently it was necessary to remove the alcohol by evaporating the 10 ml. portions to dryness on a water-bath before titrating. In this case 20 ml. of water were added in place of the 10 ml. which were used with the aqueous solutions.

The method used in determining the excess quinine in the filtrate depends upon the oxidation of the vinyl group of the quinine molecule. This method, like other permanganate oxidation

methods, required some practice before proficiency could be attained. It was found that both the degree of acidity and the temperature of the quinine solution to be titrated were important factors in the determination. Titrating into neutral or alkaline solution was totally unsatisfactory, while titrating into hot or even warm acidic solutions resulted in high values, owing to the rupture of the nucleus by the more vigorous oxidation. It was found, however, that if the quinine solution was made distinctly acid and titrated at a temperature not exceeding 25°, consistent values could be obtained from day to day for quinine solutions of known concentrations. The oxidation progressed slowly at first, but soon accelerated and proceeded until the end-point was reached. The end-point remained visible for ½ to 2 hours, depending upon the quantity of alkaloid titrated. The permanganate solution used was standardized against known weights of quinine bisulfate and was of such concentration that 1 ml. of the solution was equivalent to 1 mg. of quinine bisulfate. All titrations were made in duplicate from a small burette with readings to 0.05 ml.

Effect of H Ion Concentration.

The effect of H ion concentration of the solution upon the adsorption of quinine may be observed from Fig. 1. The curves presented divide themselves into two distinct groups with regard to the quantity of quinine adsorbed. One group represents the efficient adsorption of the alkaloid by English fullers' earth, while the other group represents the far less efficient adsorption by norit and American fullers' earth. Curve A indicates the amount of quinine, expressed as mg. of quinine bisulfate, adsorbed from aqueous solutions by 1 gm. of English earth. This curve represents an average of values obtained from two samples of English earth, since the two samples were about equally effective adsorbents. The amount of quinine adsorbed reaches a maximum in the dilute acid solutions, and shows a marked decrease in the more acidic solutions and some indications of a decrease in the more alkaline solutions.

Curve C represents the composite values obtained from three samples of American fullers' earth. Likewise, these three samples were very similar in physical properties and adsorbed almost

identical amounts of quinine under the various conditions. The variation in the adsorptive capacity of the two types of earth for this alkaloid becomes apparent when we compare Curves A and C. Although the American earths adsorbed only about one-third as much quinine as the English earths, the general form of the two curves is very much the same. The maximum adsorption by the

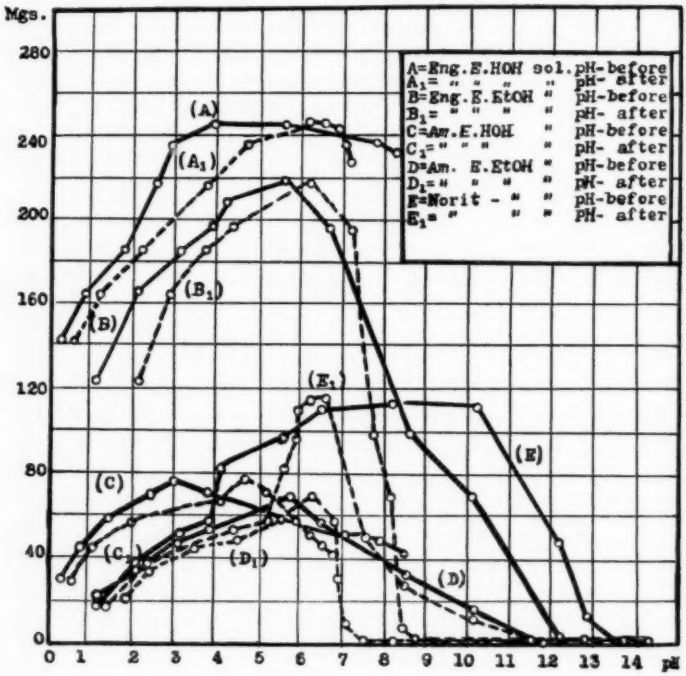

FIG. 1. Mg. of quinine bisulfate adsorbed per gm. of adsorbent at various H ion concentrations.

American earths was from slightly more acidic solutions than by the English earth. This can be explained on the basis of the difference in the composition of the two earths, as will be pointed out later.

Curve B represents the amount of quinine adsorbed by the two samples of English earth from alcoholic solutions. Although the

maximum amount of quinine adsorbed was only 88 per cent of that adsorbed from aqueous solutions of the same concentration and pH, the general trend of the two curves is very much the same. The effect of pH on adsorption from alcoholic solutions is more marked as indicated by the more abrupt rise and fall in the curve. The effect of alkalinity upon the adsorption of this substance is brought out more strikingly than was possible with the aqueous solutions.

Curve D represents the adsorption from alcoholic solutions by the American earths. These results are in full agreement with those obtained with the English earths from similar solutions. The maximum adsorption is somewhat inhibited by the alcoholic solution as was the case with the English earth, and the effect of alkalinity is equally pronounced.

Curve E shows the adsorption of quinine from alcoholic solutions by norit. This adsorbent was slightly more effective than the American earths, but removed only one-half as much quinine as was removed by an equal weight of English earth under the same condition.

The adsorption of quinine by fullers' earth appears to be a maximum just on the acid side of the isoelectric point of that earth. The slight variations in the region for maximum adsorption by different earths are due to the differences in the composition of the different earths, which result in the earths having different isoelectric points. This adsorption apparently is the result of freeing of the more active cations of the adsorbent, which in turn react with the SO_4 ions. Equilibrium is established when the less active anions of the adsorbent combine with the quinine ions to form an insoluble compound or complex.

Changes in H Ion Concentration.

On making the pH determination on the various series of filtrates, it was observed that considerable pH change resulted during the adsorption period. The magnitude of these changes varied with the adsorbent used and also with the pH of the original solution. In some cases this variation was as much as 4.5 pH units. With all unpurified adsorbents, there was a decrease of acidity in the acid solutions and a decrease of alkalinity in the alkaline solutions, or a tendency to bring the solution toward the

neutral point. With such a change of acidity or alkalinity occurring during adsorption, it became apparent that the equilibrium H ion concentration (H ion concentration of the filtrate) and not the initial pH of the solution is the determining factor.

The relation between the initial and the final reaction is shown in Fig. 1; Curves A_1, B_1, C_1, D_1, and E_1 are plotted against the equilibrium H ion concentration. The effect of alkalinity upon the adsorption of this alkaloid becomes even more marked when the data are presented in this manner.

Adsorption of Other Ions.

To ascertain what portion of the pH change could be attributed to the removal of the quinine from the solution by adsorption, and to determine the variation in the Na and SO_4 ions which accompany this adsorption, the following experiment was carried out. A series of aqueous solutions of H_2SO_4 and NaOH, ranging in pH from that of M H_2SO_4 to that of approximately 2 M NaOH, was prepared. The pH of these solutions was accurately determined, and 50 ml. of each solution were treated in a similar manner to that involved in the quinine adsorption. The pH of the resultant series of filtrates was determined for each earth. The same procedure was afterwards repeated by using in each 1 gm. of earth and 250 ml. of solution. The SO_4 content of the four most acidic solutions was determined accurately before and after the fullers' earth treatment. Likewise, the Na content of the four most alkaline solutions was determined before and after the treatment. The differences were attributed to the exchanges having taken place between the earth and the solution. The data obtained are presented in Table I.

It is evident from these data that the pH change during adsorption is due chiefly to the adsorption of the cations, as the SO_4 concentration of the filtrate shows no consistent variation from the original solution, while the concentration of both H and Na ions is decreased. This indicates that the adsorptions of ions by this adsorbent is brought about by an exchange of ions between the adsorbate and the adsorbent, and does not involve the whole molecule. Those ions which combine with the various constituents of the adsorbent to form insoluble products, and are removed from the system by filtering or other mechanical means, are con-

sidered adsorbed. The basic ions removed from the adsorbent by the sulfuric acid, react to form soluble sulfates; consequently no SO_4 ions are removed from the system when the insoluble adsorbent is removed. Cations like H and Na react to form insoluble silicates, which result in these ions being removed from the system.

This fact becomes more evident when we compare the adsorption of these ions by the electrodialyzed and the untreated earth. The process of electrodialysis will be discussed elsewhere. This treatment had little effect upon the adsorption of the anion but brought about a significant alteration in the amounts of both cations investigated. The electrodialyzed earth adsorbed considerably less H ion than the untreated earth, while it adsorbed 2.5 times as many Na ions as a similar weight of earth before treatment. This is to be expected, since by electrodialyzing fullers' earth many of its basic components are replaced by equivalent quantities of H ions, and the resultant product is more nearly saturated with H ions; consequently, less H ions can be adsorbed. This treatment,

TABLE I.

Mg. Equivalent of H, SO_4, and Na Ions Adsorbed by 1 Gm. of Fullers' Earth.

Ions.	Approximate concentration of ions.	Mg. equivalent.	
		By untreated earth.	By electrodialyzed earth.
	M		
H*	2	34.80	27.84
	0.2	24.96	14.04
	0.02	1.92	1.26
	0.002	0.32	0.16
SO_4†	1	0.25	0.27
	0.1	0.05	0.11
	0.01	0.01	0.00
	0.001	0.07	0.04
Na†	2	3.54	9.15
	0.2	2.29	2.80
	0.02	0.45	0.91
	0.002	0.23	0.30

* Calculated from degree of dissociation and pH change.
† Determined by quantitative method.

as would be expected, has the opposite effect upon the adsorption of Na ions. The electrodialyzed earth, being more nearly depleted of its basic ions, has a greater adsorptive capacity for Na ions than does the untreated earth.

Effect of Treating Earth.

In testing the adsorptive capacity of the different fullers' earths it became evident that certain earths had a much greater adsorption capacity for quinine than other earths. We, like others, became interested to know what factor or factors determined the magnitude of this difference. The first factor investigated was the degree of fineness of the fullers' earth used. A quantity of the earth was sieved by a series of seven sieves ranging from 20 to 300 meshes per inch. The amount of quinine bisulfate adsorbed by each size of particle from an aqueous solution at a pH of 3.30 was determined.

Our first experiences with the different sizes of particles showed a marked decrease in the adsorptive capacity of the larger particles. However, with fullers' earth, which had been previously passed through a 150 mesh sieve, dampened, and allowed to dry in hard lumps, and passed through the same series of sieves no variations in the adsorptive capacity of the various sizes of particles were found. From this it was concluded that the poor adsorption with the larger particles of the original earth was in part due to the large quantity of sand and inert material which naturally would predominate in these fractions, and that the state of division of this adsorbent is of minor importance so far as quinine adsorption is concerned, provided that sufficient time is given for equilibrium to be reached. The poorer adsorption of quinine by the American earths, despite the fact that they were apparently in a finer state of division than the English earths and occupied 1.7 times the volume per unit weight, is a further indication that the size of particle is of relatively small importance in this type of adsorption.

The next factor to be investigated was the effect of previous heat treatment of the earth. 250 gm. of the 150 mesh fullers' earth were divided into five equal parts, four being heated and the fifth remaining unheated. Equal portions of the earth were heated for 30 minutes at approximately the following temperatures:

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250°, 500°, 750°, and 1000°. The amount of alkaloid adsorbed by samples of each portion was then determined. It was found that the ability of the four heated portions of earth to adsorb quinine was reduced 36.8, 71.9, 86.6, and 98.7 per cent respectively. Lloyd (7) has attributed such changes in adsorptive capacity to different degrees of hydration. If dehydration is the only change that took place when the above earth was heated, then we believe the differences in the adsorptive capacities of the two types of earth studied can be explained on this basis. It seems more plausible, however, that the decreased adsorption resulted from a conversion of the replaceable cations of the earth into a more stable form by the heating. This view is substantiated by the fact that the iron present in the earth is converted into red oxide by heating the earth. This is evident from the red coloration of the earth, which increases with a decrease in the adsorptive capacity of the heated earth.

The next step was to study the effect of electrodialysis upon the adsorptive capacity of the earths. This treatment ranged from 8 to 32 hours at an E.M.F. of 90 to 140 volts, and with 2 to 0 amperes. When the earth had dialyzed for the desired period of time, it was washed from the dialyzing cell, filtered by suction, and dried in an oven at a temperature not exceeding 60°. The dry material was passed through a 150 mesh sieve and its adsorptive capacity for quinine determined.

The effect of electrodialyzing the English earths against distilled water for 32 hours is shown by a comparison of Curves 1 and 2 (Fig. 2). Curve 1 represents the adsorption of quinine from alcoholic solutions by the original earth, when plotted on the basis of equilibrium H ion concentration. Curve 2 represents similar data obtained from the dialyzed earth. While the general trend of the two curves is very much the same, the electrodialyzed earth adsorbed the maximum quantity of quinine in a more acidic solution than the untreated earth. This is to be expected since the dialysis tended to make the earth more acidic by the removal of the water-soluble electrolyte and at least part of the replaceable bases, which resulted in the shifting of the isoelectric point of the treated earth toward the acid side of the pH range.

Of the factors investigated thus far, heating proved most effective in altering the adsorptive capacity of a given earth. When

an earth has been heated sufficiently to impair its adsorptive capacity seriously, a reddish coloration of the earth resulted. This coloration suggested the presence of iron compounds. Since the English earths which were the most effective as an adsorbent of quinine likewise showed the most color on being heated, this sug-

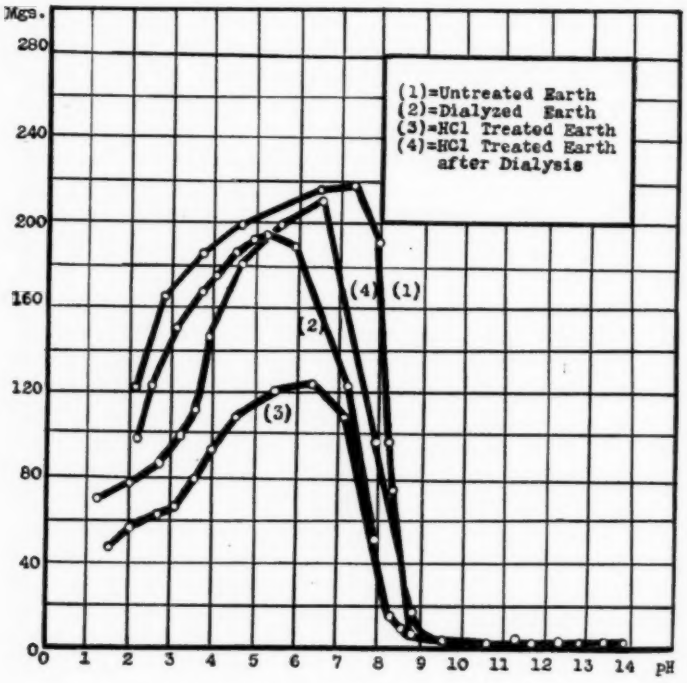

FIG. 2. Mg. quinine bisulfate adsorbed by treated fullers' earth from aqueous alcohol at various equilibrium pH values.

gested the possibility that the iron compounds were related to adsorption of the alkaloid, consequently a sample of English earth was digested in boiling 10 per cent HCl. The earth was filtered out and the treatment repeated for a total of five extractions. The residue was washed with distilled H_2O until the filtrate was free from chlorides and was then dried at a low temperature. The

dried material was then passed through a 150 mesh sieve and its adsorptive capacity for quinine determined.

Curve 3 (Fig. 2) indicates the adsorption of quinine by the earth after the treatment with the hot acid. There was a loss of about 48 per cent of the original adsorptive capacity of the material. This adsorbent had a much greater effect on the reaction of the alkaline solutions than the original material. The maximum adsorption of quinine occurred in the solution having an initial pH of 10.26 and an equilibrium pH of 5.6. Whether the decreased acidity was due to adsorption of Na ions or to neutralization of Na by adsorbed Cl ions was not determined.

A portion of the acid-treated earth was electrodialyzed until the diffusible ions were exhausted. Curve 4 (Fig. 2) shows the ability of this material to adsorb quinine. The adsorption by the acid-treated and dialyzed earth was nearly as effective as the adsorption by the original earth. It was noted that during the early stages of the dialyzing a considerable quantity of chlorine was liberated from the anode chamber; this was followed by the appearance of hydrochloric acid in the anode chamber and basic ions in the cathode chamber. This phenomenon suggested the possibility of the replacement of OH groups from the earth by Cl ions during the drastic acid treatment; such a reaction would result in the formation of a chlorosilicate complex and decrease the potential store of exchangeable cations (H ions) in the adsorbent.

In order that a study might be made of the adsorptive properties of the substances removed from the fullers' earth, the combined filtrates and washings were placed in a large evaporating dish and brought to pH 6.8 with NaOH. A reddish gelatinous precipitate formed which was removed, after the solution became cool, by filtering with suction. This red precipitate was washed until the filtrate gave a negative chloride test and then dried. The filtrates and washings were combined and made distinctly alkaline with ammonium hydroxide. A grayish gelatinous precipitate formed, which was treated in a similar manner as the reddish precipitate.

The precipitated Fe_2O_3 and Al_2O_3 were electrodialyzed until free of diffusible ions and their adsorptive capacities for quinine determined. There was practically no adsorption of quinine by either adsorbent in the acid solutions and only a very slight adsorption in the alkaline solutions.

Adsorption of Oxalate.

Experimental Procedure.

4 gm. of oxalic acid were dissolved in 750 ml. of distilled water, the acidity or alkalinity adjusted to approximately the desired pH

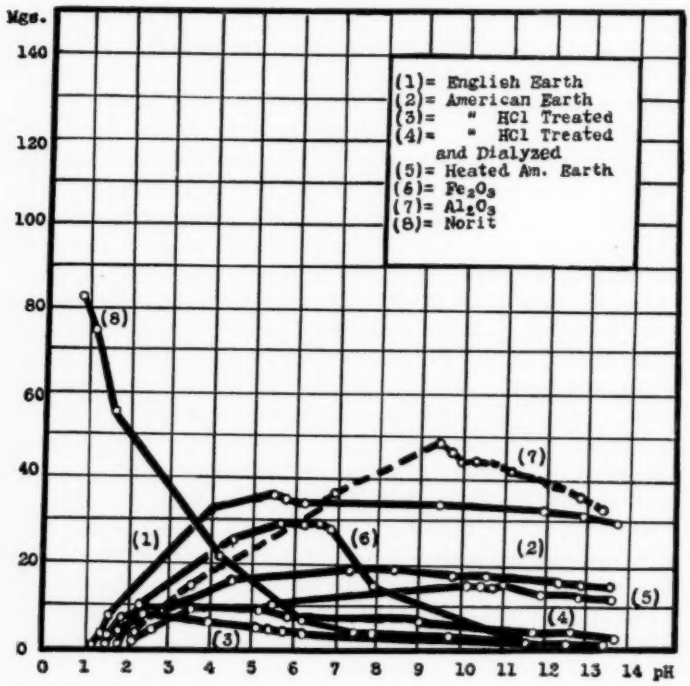

FIG. 3. Mg. of oxalate adsorbed per gm. of adsorbent from aqueous solutions at various equilibrium H ion concentrations.

with H_2SO_4 or NaOH, and the volume increased to 1000 ml. After the solution was mixed thoroughly, the H ion concentration and the oxalate content were determined. A series of twelve solutions was prepared in this manner, ranging in pH from 0.98 to 13.53 and containing the equivalent of 4 mg. of oxalic acid per ml.

For the adsorption measurement, 50 ml. of solution and 1 gm.

of the adsorbent were placed in a dry 100 ml. flask, shaken for 1 hour, and filtered through a small quantitative filter into a dry receptable. H ion determination was made on a small portion of the filtrate. The unadsorbed oxalate was determined by titrating duplicate 10 ml. portions of the filtrate with standard permanganate solution. Blank determinations were made upon original solutions, by putting them through a similar procedure but omitting the adsorbent.

Effect of H Ion Concentration.

The effect of H ion concentration upon the adsorption of oxalate ion is illustrated in Fig. 3. Curve 1 shows the adsorption of oxalate from aqueous solutions of various H ion concentrations by English fullers' earth. The maximum adsorption occurred in the region of the neutral point. There was not the sharp decrease in adsorption on either side of this point, which was noted on each side of the optimal pH in the quinine adsorption. There was, however, a pronounced inhibiting action in the solutions more acid than pH 4.0, the adsorption reaching 0 around pH 1.0. The effect of alkaline solutions was less pronounced. Curve 2 represents the adsorption of oxalate ion by American fullers' earth. This adsorbent was less effective than the English earth but the effect of the reaction of the solution upon the adsorption is comparable.

Curve 3 indicates the adsorption of oxalate by the American earth after it had been treated with hot hydrochloric acid, and Curve 4 shows the effect of electrodialyzing the acid-treated earth. It is evident that the acid treatment almost destroyed the power of the earth to adsorb the oxalate ion and that this ability was not restored by the electrodialyzing process.

The effect of heating the American earth for 30 minutes at approximately 1000° is shown in Curve 5. This treatment depressed the adsorption, particularly in the acidic solutions. In the stronger alkaline solutions there was a very slight depression of the adsorption owing to the previous heating of the adsorbent.

Curves 6 and 7 show the adsorption of oxalate by the iron and aluminum preparations which were obtained in the previously described treatment of the fullers' earth with hydrochloric acid. The adsorption by these substances in the acid region was quite similar to that by the original earth from which they came. The

ferric oxide adsorbent showed an optimum adsorption in the region of pH 5.0 to 7.0 and rapidly decreased in efficiency with further increase in alkalinity. The aluminum preparation on the other hand, increased in efficiency until a pH of about 9.5 was reached and decreased very gradually in the more alkaline solutions.

Norit was the most efficient of the substances tested as adsorbents for oxalate. Curve 8 shows that the effect of H ion upon the adsorption of oxalate by norit was quite different from its effect upon the adsorption by the other adsorbents; the most efficient adsorption by norit occurred in the most acidic solutions used and declined rapidly with decrease in H ion concentration.

It seems evident from the data presented that the adsorption of oxalate by fullers' earth depends upon the basic substances contained by the earth and not upon the silicate part of the complex. This is indicated by the greater efficiency of the English earth which analyses showed to contain more basic substances than the American earth. It is further shown by the fact that removal of the bases by acid treatment almost completely destroyed the ability of the earth to adsorb oxalate. Moreover, the oxides of iron and aluminum which were removed from the English earth were found to be active adsorbents of oxalate. It is apparent that the adsorption of oxalate by fullers' earth is simply the formation of insoluble oxalates with certain bases of the adsorbent; anything which favors the formation of these insoluble oxalates will, therefore, increase the apparent adsorption of oxalate ion. The decrease in adsorption of oxalate with increase in the concentration of H ion is apparently a reflection of increasing solubility of the oxalates formed.

Adsorption of Glucose.

Experimental Procedure.

A series of aqueous solutions of glucose was prepared, ranging in pH from 0.87 to 12.46 and containing 10 gm. of purified glucose per liter. Solutions of glucose, more alkaline than 12.45, were prepared but were not used because of the destruction which became apparent through the brown coloration which formed. This coloration was not observed in the acidic solutions, or alkaline solutions with a pH less than 12.46, even after they had stood

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several days. For the adsorption, 1 gm. of the adsorbent was weighed accurately and placed in a 100 ml. Erlenmeyer flask and 50 cc. of the solution added. The flask was shaken from time to time during a period of 1 hour, and filtered through a small filter into a dry 100 ml. volumetric flask. A small portion of this fil-

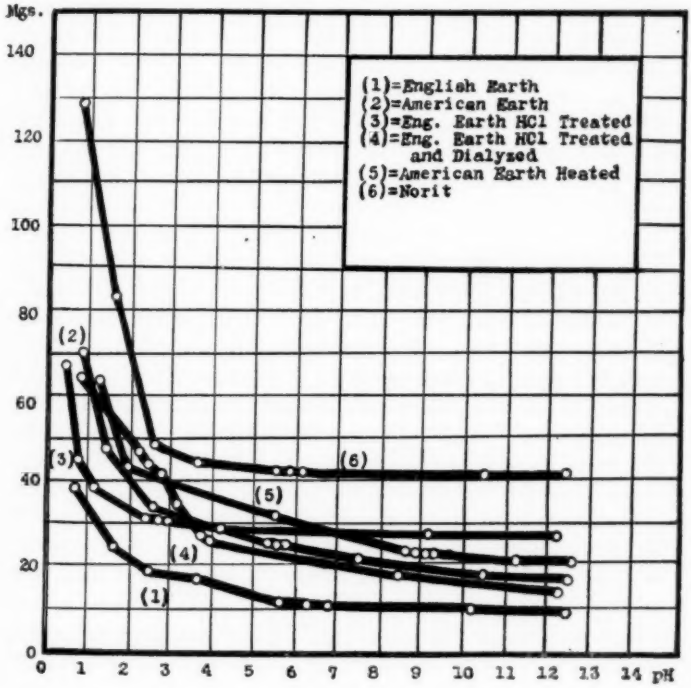

FIG. 4. Mg. of glucose adsorbed per gm. adsorbent from aqueous solutions at various equilibrium H ion concentrations.

trate was used in the pH determination. The unadsorbed glucose was determined by the Benedict (24) method. The amount of glucose adsorbed by a number of adsorbents was determined by this method. Blank determinations were made upon all solutions previous to the treatment with the adsorbents.

Effect of H Ion Concentration.

Fig. 4 shows the effect of the H ion concentration on the adsorption of glucose from aqueous solutions by fullers' earth and norit. Curve 1 represents the weight in mg. of the glucose adsorbed by 1 gm. of English earth at the various equilibrium H ion concentrations. The greatest adsorption of this substance was obtained in the most acidic solutions. This is not in agreement with the findings of Hauge and Williaman (23); however, the solutions employed by us were considerably more acidic than the ones used by these investigators. Another factor which might have led to different results is that our solutions contained twice as much glucose as the solution used by Hauge and Williaman. The adsorption of this substance in acidic solutions probably is affected by at least two important factors. The first of these factors is the solubility of glucose in strongly acidic solutions. Since glucose is a slightly acidic substance, its solubility in strongly acidic solutions is less than in water or dilute sodium hydroxide solutions. This decreased solubility in strongly acidic solutions results in a more favorable condition for adsorption. The second factor is the effect of acid on the surface tension of glucose solution. Glucose, when dissolved in distilled water, has very little effect upon its surface tension, but the same amount of glucose dissolved in dilute sulfuric acid has a very outstanding effect upon the surface tension of the resulting solution. When 10 gm. of glucose were dissolved in 1 liter of distilled water at 26°, its surface tension was raised 0.5 dyne, while the same weight of glucose dissolved in an equal volume of M sulfuric acid at the same temperature resulted in the lowering of the surface tension by 15.8 dynes. This lowering of surface tension creates a condition most favorable for adsorption.

Curve 2 shows the adsorption of glucose by a sample of American earth. This earth was in a much finer state of division than the English earth, and consequently had a greater specific surface. This being true, a greater adsorption of a non-polar substance is to be expected. Curve 3 shows the adsorption by a sample of the English earth after having received the hydrochloric acid treatment. Such a treatment always resulted in a more acidic product and in a higher degree of fineness than the original earth.

Both of these alterations are evident from the adsorption curve. Curve 4 shows the adsorption by the acid-treated earth after being electrodialyzed. It is apparent that electrodialysis did not affect the adsorption by this substance other than to reduce its acidity.

Curve 5 shows the adsorption by the American earth after being heated. Evidently, the only detrimental effect of heating the earth upon the adsorption of glucose is that the resultant product is always more alkaline than the unheated earth. This tends to shift the pH of the solution farther toward the alkaline side which results in a slight decrease in adsorption in the more acidic solutions.

Curve 6 shows the adsorption of glucose from the same series of solutions by norit. It is evident from this curve that the adsorption by this substance is affected by the same factors as those affecting the adsorption of glucose by fullers' earth. The greater adsorption by this adsorbent is evidently due to its greater specific surface.

DISCUSSION.

It is recognized that the data presented in the preceding pages are by no means complete. Nevertheless, the results obtained have afforded helpful suggestions for the control of conditions in the adsorption of the active factors of the vitamin B complex. Some of the results likewise seem to have general application to adsorption problems.

The most important factor affecting the efficacy of fullers' earth as an adsorbent is the nature of the earth itself. The problem why one variety of earth is an active adsorbent and another variety practically worthless, has never been completely solved. The present studies have shown, however, that the relative efficiency of different earths varies with the character of the substance to be adsorbed. The ability of an earth to adsorb alkaloids or other basic substances probably depends upon the ability of the silicate group of the complex to ionize and bind cations. This property no doubt is closely related to the degree of hydration which was pointed out by Lloyd as a requisite of adsorption by this agent. The adsorptive capacity of an earth for anions, on the other hand, is a direct result of the formation of insoluble compounds of the anions with the basic ions of the adsorbent. The efficiency of an earth in this type of adsorption is measured,

therefore, by its content of bases which are capable of forming insoluble complexes with a given anion. The adsorption of a non-electrolyte is apparently not related to the ionization of the earth but is a function of the specific surface of the adsorbent. The English earths are apparently more efficient than the American earths for the adsorption of electrolytes; the American earths, however, excel as adsorbents for non-electrolytes of the glucose type. The English earths used in these studies weigh about 1.75 times as much per unit volume as the American earths; this indicates a smaller specific surface for the former and probably explains their less efficient adsorption of glucose.

The effect of various treatments upon the adsorptive capacity of fullers' earth, likewise, varies with the type of substance to be adsorbed. Heating of an earth for 30 minutes at about 1000° almost completely destroys the ability to adsorb quinine; this treatment slightly impairs the ability to adsorb oxalate; the ability to adsorb glucose, however, is not affected significantly by this degree of heating. The heating no doubt dehydrates the salts of silicic acid and thus results in the formation of the stable oxide which cannot be hydrated again under the conditions in which it was tried as an adsorbent.

A surprising result is that electrodialysis of the English earth decreases its efficiency as an adsorbent of quinine. Since the basic substances in the natural earth do not adsorb quinine, it seems logical to expect that their removal would increase the efficiency of the purified residue. This was not found to be true but the explanation is not apparent.

Treatment of English fullers' earth with hot hydrochloric acid greatly reduces its ability to adsorb quinine, almost completely destroys its ability to adsorb oxalate, but increases its efficiency as an adsorbent for glucose. This treatment removes nearly all the bases from the silicate complex and replaces them with hydrogen. There is a further possibility that the treatment is sufficiently drastic to cause the silicic acid thus formed to give up some OH ions for which Cl ions are substituted. In each group thus formed the possibility of an exchange of H ions for any other cation would be lost. The escape of chlorine gas at the anode and the restoration of the efficiency of the acid-treated earth upon electrodialysis seem significant in this connection. Since the bases

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in the crude fullers' earth are responsible for the adsorption of oxalate, it is evident that their removal by any treatment would prevent the adsorption of this ion. The increased adsorption of glucose is probably due largely to an increased dispersion of the adsorbent and to the fact that the acid-treated adsorbent has less tendency to decrease the H ion concentration of the solutions.

One of the most important controllable factors in adsorption by fullers' earth is the H ion concentration of the solution in which the adsorption takes place. While the present studies have been definitely related to the concentration of H ion, the adsorption really is influenced by the concentration and relative activity of all the ions in the system. If fullers' earth is treated with a solution containing a mineral acid, the bases are removed from the earth and replaced by their equivalent of hydrogen until equilibrium is established. The bases are removed in the order of their position in the electromotive series; the higher the element in the series, the more easily is it replaced. If the process is continued far enough, all of the basic ions will be replaced by H ions. Now if the acid adsorbent is placed in an alkaline solution the reverse reaction occurs; the basic ions replace the H ions until an equilibrium is established. On the acid side of what may be termed the isoelectric point of the earth the tendency is toward the adsorption of the weaker cations but on the alkaline side of this point the tendency is toward the adsorption of the stronger cations; the reaction is favored in the former case by the formation of the weakly ionized silicic acid and in the latter case by the formation of H_2O as one of the end-products.

It is evident, therefore, that a weak basic ion can more readily compete with H ions in an acid medium than can a strong basic ion; hence the weaker the cation, the greater is the concentration of H ion favoring its optimal adsorption.

The adsorption of anions by fullers' earth may likewise be considered as a competition between the various cations of the system, the same factors affecting the pairing of ions as are operative in the adsorption of cations. It is evident that the maximum adsorption of a given anion will occur under conditions in which those cations of the system, which form soluble compounds with the given anion, replace from the fullers' earth those cations which form insoluble compounds with the anion to be adsorbed. The

formation of these insoluble compounds is obviously a requisite of adsorption.

The adsorption of non-electrolytes apparently is not affected by the ionization of the adsorbent. The H ion concentration of the solution, nevertheless, may play an important part in the adsorption of such substances. In this type of adsorption the importance of solubility of the substance to be adsorbed and the effect of viscosity and surface tension of the solution are at a maximum. In so far as H ion concentration affects these phenomena, it will also affect the adsorption of substances like glucose.

The optimal adsorption of quinine and of glucose by norit occurs at the same H ion concentration which favors their optimal adsorption by fullers' earth. The effect of H ion concentration upon the adsorption of oxalate is quite different for the two adsorbents. The adsorption of this compound by norit gradually decreases from pH 0.98 to pH 7.0 and is almost negligible throughout the alkaline range. It is possible that the adsorption of quinine by norit is identical in nature with its adsorption by fullers' earth; in the case of norit there probably is an exchange of cations from inorganic impurities in the adsorbent for quinine ions. As a consequence of the low inorganic content of norit, it is a much poorer adsorbent for quinine than is fullers' earth. Norit is, however, a better adsorbent for oxalate than is fullers' earth; this is especially true at the higher H ion concentrations. It is apparent that norit, unlike the bases of fullers' earth, can form compounds with oxalate which are insoluble in the most acidic solutions used in these tests.

SUMMARY.

1. A study of the effect of certain factors upon the adsorption of quinine, oxalate, and glucose by fullers' earth and norit has been made.

2. Different fullers' earths have different adsorptive capacities because of differences in both composition and state of division.

3. H ion concentration has been found to play an important rôle in the adsorption of electrolytes, and under certain conditions, it also affects the adsorption of non-electrolytes.

4. Degree of dispersion of adsorbent is more important in apolar adsorption than in polar adsorption.

5. By heating fullers' earth at a red heat for 30 minutes, its adsorptive capacity for both oxalate and glucose is only slightly altered.

6. The electrodialyzing of fullers' earth slightly reduces its adsorptive capacity for quinine.

7. Fullers' earth which has been previously digested with hot mineral acid, adsorbs only one-half as much quinine as the untreated earth, while it adsorbs only one-sixth as much oxalate as before the treatment. Such treatment has little effect upon the adsorption of glucose by this adsorbent.

8. Electrodialysis of the acid-treated earth restores much of its quinine adsorptive capacity but has little effect upon the adsorption of either oxalate or glucose.

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THE EFFECT OF HYDROGEN ION CONCENTRATION UPON ADSORPTION OF THE ACTIVE FACTORS OF VITAMIN B COMPLEX BY FULLERS' EARTH.*

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Fullers' earth has been used as an adsorbent for vitamin B by several investigators (1–7). In general the practice has been to adsorb the vitamin from acidulated extracts of various plant substances. Either the activated fullers' earth was then fed as a source of the vitamin, or the adsorbed substance was displaced from the adsorbent by treatment with alkaline solutions of varying strength. The methods have been more or less empiric and the reactions of the solutions used in both the adsorption and the elution processes have varied within rather wide limits. This has to a large extent been due to a lack of sufficient data regarding the influence of H ion concentration upon the adsorption process. Levene and van der Hoeven (3) have concluded that the H ion concentration for the optimal adsorption of the vitamin B complex by Lloyd's reagent as indicated by the rat growth method is at pH 4.0. Their results, however, afford no idea of any possible differences in the adsorption of the separate factors of the complex. Kinnersley and Peters (8) have reported that the optimal adsorption of the B-P factor ("torulin") by charcoal occurs at pH 7.0 and that the active substance is not adsorbed at pH 2.5. These investigators (9) reported unsatisfactory adsorption of the active factor upon fullers' earth but they do not report the reaction at which the adsorption was attempted.

Previous studies in this laboratory (6, 7) have shown promising results from the use of fullers' earth as a selective adsorbent for

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the B-P factor. It was thought possible that a strict control of hydrogen ion concentration might further increase the selective action, particularly if the two factors should have different optima. In the accompanying paper, Guerrant and Salmon (10) report the effect of H ion concentration upon the adsorption of an organic cation, anion, and also of a non-electrolyte by fullers' earth. These studies emphasize further the relation between reaction of the solution and efficiency of adsorption by fullers' earth.

A study of the effect of H ion concentration upon the adsorption of the B-P and the P-P factors has yielded results which are deemed important in the development of methods for concentrating these factors. An account of the study is presented in the following pages.

Preparation of Adsorbates from Extract of Maize.

White maize of the current year's crop was dried at 45–50° and finely ground. The ground material was placed in large percolators and extracted at room temperature with 80 per cent (by weight) alcohol. The percolation was continued until 225 liters of extract were obtained from 150 kilos of maize. This extract was then reduced by vacuum distillation to a volume of about 15 liters, allowed to cool, and the mass of precipitated protein was removed. The protein was kneaded thoroughly to remove as much as possible of the occluded extract which was poured off and the protein again kneaded in three successive portions of water. The washings were added to the extract and the total volume made up to 21.5 liters with H_2O. The volume was then reduced as before to about 15 liters to insure complete removal of the alcohol. The extract was kept in an electric refrigerator for about 48 hours and was then filtered by suction and diluted to 16.5 liters. The filtrate was a clear yellowish fluid with a surface tension of 55 dynes, a pH of 4.70, and a sp. gr. of 1.005. This solution was divided into ten portions, the first four representing 10 kilos of maize each, the next two 15 kilos each, and last four 20 kilos each. The reaction of the various portions was adjusted with H_2SO_4 or NaOH to the following respective values: pH = 0.54, 1.56, 2.73, 4.16, 5.64, 6.81, 8.15, 9.59, 10.57, 11.89. All pH determinations were made with the calomel electrode. The final volume of each portion was kept proportional to the amount

of original maize it represented. After the pH adjustments had been made, surface tension, viscosity, and specific gravity measurements were taken. The various portions were then treated with 3 gm. of English fullers' earth per kilo of maize, each solution being stirred vigorously at 5 minute intervals for a period of 2 hours. The adsorbates (activated solids) were then removed on Buchner funnels and washed twice with 25 ml. of 80 per cent alcohol per 30 gm. of solid and twice with the same amount of 93 per cent alcohol. The various portions of the adsorbate were partially dried at room temperature, pulverized, and placed in vacuum desiccators until completely dry.

The washings from the adsorbates were freed of alcohol by vacuum distillation and returned to their respective filtrates. The pH, surface tension, and viscosity were again determined on each filtrate. After the alkaline filtrates were made distinctly acid to litmus, all filtrates were allowed to stand overnight. The reaction of each filtrate was then adjusted to approximately pH 4.0 and the concentration of Na_2SO_4 equalized by the addition of solid Na_2SO_4 where necessary. Each solution was again treated with the same amount of fullers' earth as before, the procedure being identical for the two treatments, except the pH of the solutions which had been readjusted as mentioned.

Preparation of Adsorbates from Extract of Yeast.

The yeast was extracted in portions of 2.5 to 5.0 kilos and the concentrated extract divided into four or five portions which were treated with fuller's earth at different H ion concentrations. Since the procedure was uniform for all the preparations, the method for one lot is described as typical of the series.

Yeast 1.—8 liters of H_2O acidulated with 0.75 per cent by volume of glacial acetic acid were heated to 95°. A 2.5 kilo portion of dry bakers' yeast was then stirred in and the mass thoroughly agitated. The mixture was allowed to cool to 60° and then received an addition of 12 liters of 93 per cent alcohol which flocculated the protein and facilitated the filtering process. The coagulate was filtered out on Buchner funnels and again extracted with 4 liters of warm 51 per cent alcohol. The combined filtrates were concentrated by vacuum distillation to 6.6 liters. After having stood in a refrigerator overnight the concentrate

was filtered and divided into four aliquots. The pH adjustments were made by the use of H_2SO_4 or NaOH, and 160 gm. of American fullers' earth per kilo of yeast were added. The mixtures were agitated intermittently for 2 hours. The adsorbates were then filtered out and each portion washed with four 25 ml. portions of H_2O and one 50 ml. portion of 93 per cent alcohol. They were then dried as in the maize preparations.

The washings were combined with their corresponding filtrates and each portion readjusted to the original pH of the concentrated extract. Each portion was then concentrated by vacuum distillation. When the volume had been reduced to 100 to 200 ml., sufficient alcohol was added to give a 51 per cent alcoholic solution (by weight). The precipitate of inorganic salts was filtered out and washed with three 50 ml. portions of 51 per cent alcohol. The filtrate and washings were again concentrated as before. When the solution reached a sirupy consistency it was poured upon 0.32 gm. of Argo corn-starch per gm. of yeast represented. The mixture was dried in an oven at 50–55°, ground, sieved, and stored in glass containers. These evaporated filtrates are the residues which will be referred to in the growth experiments.

Yeast 2.—The procedure was the same as for Yeast 1 except that only fifteen-seventeenths as much fullers' earth was used per unit of yeast. This variation was made because previous tests had shown Yeast 2 to be only fifteen-seventeenths as potent in B-P activity as Yeast 1.

Effect of pH upon Adsorption of the B-P Factor.

The B-P activity of the adsorbates from the maize and the yeast preparations was determined by establishing the minimum dosage required to protect completely pigeons weighing 300 to 350 gm. The essentials of the method have been described elsewhere (6). In the present experiments each bird received a total of 16.0 gm. of the basal diet per day. The adsorbates from the corn preparations were fed in double doses on alternate days but those from the yeast preparations, being much less potent, were fed daily.

The effect of pH upon adsorption of the B-P factor is illustrated by Curves A and B, Fig. 1. On the basis of the pH of the extract before adsorption, the optimal adsorption occurred at pH 2.73.

XUM

The protective dose of the resulting adsorbate was 0.0525 gm. per day. Thus 100 gm. of the solid carried 1904 protective doses, which is about 14 per cent less than the sample of earth used will

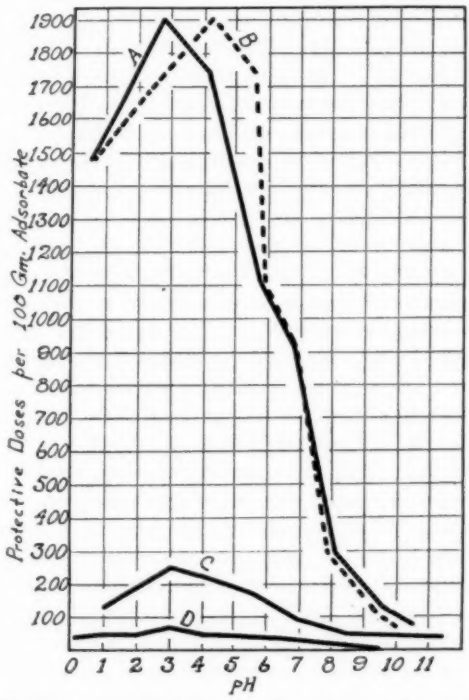

FIG. 1. Effect of pH upon adsorption of B-P factor. Curve A, protective doses per 100 gm. of adsorbates from maize, based on pH of extracts before adsorption. Curve B, same except based on pH of filtrates after adsorption. Curve C, protective doses per 100 gm. of adsorbates from Yeast 1, pH before adsorption. Curve D, protective doses per 100 gm. of adsorbates from Yeast 2, pH before adsorption.

carry when saturated with the B-P factor. The adsorption at pH 0.54 (before adsorption) was 77 per cent; at pH 1.56, 87 per cent; at pH 4.16, 91 per cent; and at pH 5.64, 58 per cent of the optimum and continued to decrease rapidly as the alkalinity in-

creased. In the acid solutions there was apparently an exchange of H ions for basic ions from the fullers' earth and a consequent shift of the pH of the solution toward the neutral point. As a result of this the extract at pH 2.73 before adsorption showed a value of pH 4.2 after adsorption while the one at pH 4.6 shifted to pH 5.6. The pH changes of the other extracts were of considerably less magnitude. The data indicate that there is a zone of maximum adsorption between pH 3.0 and pH 5.5 and that the optimal adsorption may be obtained by holding the reaction constant at pH 4.0 during the adsorption process or by having the solution sufficiently acid that the reaction is not raised above pH 4.0 during this process.

In order to determine whether any of the B-P factor was destroyed by exposure to the alkali, the B-P activity of several of the adsorbates from the second adsorption was determined. There was evidence of some inactivation of the B-P factor beginning at pH 6.81 and increasing significantly throughout the alkaline range. The poor initial adsorption in the alkaline solutions could not be explained on the basis of destruction, since at equilibrium pH 10.0 in the initial adsorption only 80 protective doses of B-P factor per 100 gm. of adsorbent were removed, yet the second adsorption showed that 834 adsorbable protective doses remained in the filtrate from the initial adsorption. Nevertheless, this indicates a loss of 977 protective doses or 51 per cent of the original adsorbable substance, if it is assumed that the sodium sulfate did not decrease the efficiency of the second adsorption. A loss of 51 per cent of the activity by such a short exposure to a weak alkaline solution is quite serious in view of the fact that alkaline solutions seem to offer the best medium for replacement of the adsorbed substance from the fullers' earth. It emphasizes the importance of using a weak alkaline solution and the shortest possible exposure to the alkali in the replacement process.

The optimal adsorption of B-P factor from the yeast extracts was obtained in the extracts having a reaction of about pH 3.00 before adsorption (Curves C and D, Fig. 1). (Unfortunately determinations of pH after adsorption were made on only a few of the yeast extracts.) The trend of the adsorption curves for the yeast extracts is very similar to that of the maize extract. The total adsorption at the optimal pH and the differences in the

amount of the B-P factor adsorbed at different pH values are very much less, however, than in the case of the maize preparations. These differences may be explained in part by the large amount of fullers' earth used for the yeast extracts. Previous tests have shown that the sample of fullers' earth used for the yeast preparations is able to adsorb 1000 protective doses per 100 gm. of earth. The most efficient activated solid from the yeast preparation (prepared from Yeast 1 at pH 3.06) carried only 250 protective doses. It is apparent that the concentration of B-P factor in the yeast extract was only sufficient for 25 per cent of saturation of the fullers' earth at the optimal pH. The effect of pH on adsorption of the B-P factor doubtless would have been more pronounced with sufficient of the active factor in solution for complete saturation of the solid at the optimal pH. The results from Yeast 2 are in agreement with this view. The most active solid (adsorption pH = 3.0) from this sample contained only 71 protective doses and the adsorption curve for this sample is even flatter than the curve for Yeast 1.

A comparison of the behavior of the two yeasts shows some rather striking differences. The amount of Yeast 1 used for 100 gm. of fullers' earth carried 416 protective doses of B-P factor. From this amount 250 protective doses per 100 gm. of solid were recovered at pH 3.0, giving a recovery of 60 per cent of the original activity of the yeast. The amount of Yeast 2 used per 100 gm. of fullers' earth represented the same number of protective doses. Nevertheless, only 71 protective doses, 17.6 per cent of the original activity, were recovered at the optimal pH. Tests with rats failed to demonstrate any B-P factor remaining in the residue (filtrate) from the pH 3.0 adsorption of Yeast 2 but did indicate the presence of slight B-P activity in the corresponding residue from Yeast 1. It seems probable that the discrepancy was due to incomplete extraction of the active material from Yeast 2.

Effect of pH upon Adsorption of the P-P Factor.

The method of determining the P-P activity of the various preparations from the yeast was essentially the same as used in previous tests in this laboratory (6, 7). Rats (45 to 50 gm.) received Diet 2 B for a preliminary depletion period of 2 weeks. They then received 0.03 gm. per day of B-P Solid 25 as a source

of the B-P factor. This was prepared from an alcoholic extract of maize by the method described in the preceding pages, except that the concentrated extract was adjusted to pH 4.0 and treated with 2.5 gm. of English fullers' earth per kilo of corn. This adsorbate in doses of 0.03 gm. daily furnishes sufficient B-P factor for growth but an insufficiency of P-P factor either to produce growth or to prevent the onset of characteristic symptoms of pellagra. In addition to Diet 2 B supplemented by B-P Solid 25, daily doses of 0.20 gm. of adsorbate from the yeast or 0.25 gm. of the residue (filtrate remaining after adsorption) were fed to each rat. These amounts, even of the most active preparations used, were not adequate to support an optimum rate of growth; hence, any variations in activity would be more readily apparent.

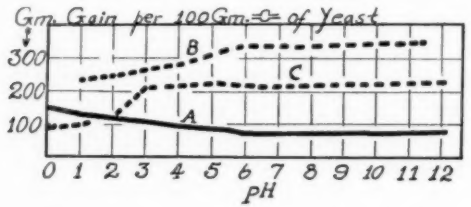

FIG. 2. Effect of pH upon adsorption of P-P factor. Curve A, gain in gm. per 100 gm. equivalent of yeast from adsorbates from Yeasts 1 and 2. Curve B, gain in gm. per 100 gm. equivalent of yeast from residues (filtrates) of Yeast 1. Curve C, same except for Yeast 2.

In order that the P-P activity of the adsorbates and the residues might be compared, the amount of growth produced in 4 weeks by the various preparations is reported on the basis of gain in gm. per 100 gm. equivalent of yeast. The results are shown in Fig. 2. Since the gains produced by the adsorbates from Yeast 1 and Yeast 2 at corresponding pH values were very similar, the results are combined for Curve A.

The most acid solution used, having a value of pH 0.08 before adsorption, yielded the adsorbate with the maximum P-P activity. The amount of active substance apparently decreased gradually from this point to pH 6.3 and remained approximately constant from the latter point to pH 12.07, the most alkaline solution used. It seemed that the P-P factor adsorbed throughout the alkaline range was about one-half that adsorbed at pH 0.08.

The activity of all of the residues was not determined; however, a sufficient number was tested to confirm the findings on the adsorbates. It is evident that the filtrates from the adsorptions in the acid region were significantly less potent in their growth-promoting action than were those from the adsorptions in the alkaline region. A comparison of Curves B and C, Fig. 2, shows that a much greater amount of P-P factor was extracted from Yeast 1 than from Yeast 2. The most potent residues from the alkaline adsorption of extracts from Yeast 1 produced about 340 gm. gain per 100 gm. equivalent of yeast. On the other hand the most active residue from the preparations of Yeast 2 produced only 220 gm. gain per 100 gm. equivalent.

Although the greater activity of the residues from Yeast 1 indicates that there was a notably higher concentration of the P-P factor in the extracts from Yeast 1 than from Yeast 2, this difference was not reflected in the activity of the adsorbates prepared from the two sources. It seems, then, that the potency of the adsorbates was limited by the ability of the fullers' earth to adsorb the P-P factor. This indicates that fullers' earth is not an efficient adsorbent for the P-P factor and suggests the desirability of a better adsorbent. Further work along this line is contemplated.

DISCUSSION.

It is apparent that the reaction of the solution determines to a large extent the efficiency of adsorption of either the B-P factor or the P-P factor by fullers' earth. There is a significant difference, however, in the effect of pH upon the adsorption of the two factors. The optimal adsorption of the B-P factor from extracts of yeast or maize was obtained in a zone between pH 3.0 and pH 5.5 (equilibrium pH) with the optimal point at about pH 4.0. From this zone there was a gradual decrease in adsorption with increase in acidity and a rather rapid decrease with increase in alkalinity. The adsorption practically reached zero at about pH 11.0. On the other hand the optimal adsorption of the P-P factor occurred at pH 0.08 the most acid solution tested. There was a gradual decrease in adsorption to pH 6.3, from which point the adsorption remained practically constant throughout the alkaline range of the tests.

It is interesting to compare the adsorption of the two factors with the adsorption of quinine and of glucose as reported by Guerrant and Salmon (10). The effect of pH upon adsorption of the B-P factor is very similar to its effect upon the adsorption of quinine; again in the adsorption of the P-P factor and of glucose, there is a comparable effect of pH. Another similarity in the relative amounts of quinine and of glucose adsorbed is found in the apparent relative adsorption of the B-P and the P-P factors. Although it is not possible to determine the absolute amounts of the two factors adsorbed, there are reasons for believing that the adsorption of B-P factor by fullers' earth is much greater than the adsorption of P-P factor. One indication of this is the relative completeness of the removal of the two factors from solution, it being far more difficult to deplete an extract of its P-P activity than of its B-P activity. Again the efficiency of the respective adsorbates seems to indicate a greater adsorption of the B-P factor: it is possible to prepare an adsorbate which has many times the B-P activity of the most potent natural sources of this factor; in contrast with this, the adsorbates from yeast extracts as prepared in these tests were apparently not as potent sources of the P-P factor as the yeast from which they were prepared. These differences seem to suggest phenomena associated in the one case with the polar and in the other with the apolar type of adsorption.

The sensitivity of the B-P factor to an alkaline reaction indicates the importance of using a weakly alkaline eluent in the process of replacing the active factor from the adsorbate. Although it is not certain that the adsorption is reversible, it seems probable that a satisfactory recovery of the active substance may be effected by the use of an eluent having a reaction of pH 8.0 to pH 9.0.

In the above studies the P-P factor has been considered as dual in nature, having both a growth-stimulating and a pellagra-preventing action. It is by no means certain, however, that both properties are inherent in a single substance. An increasing accumulation of evidence seems to indicate that at least two substances are involved in this dual function of the P-P fraction. This introduces the possibility that the adsorption of the P-P factor as determined in these experiments represents a composite

adsorption of more than one active substance. Nevertheless, the data are submitted with the belief that they establish a basis for a more efficient utilization of fullers' earth in the separation and concentration of the components of the vitamin B complex.

SUMMARY.

1. The maximum adsorption of the B-P factor by fullers' earth was obtained in a zone between pH 3.0 and pH 5.5 (equilibrium reaction) with the optimal point at pH 4.0.

2. The adsorption of the B-P factor diminished rapidly as the alkalinity increased beyond pH 5.5, becoming negligible at pH 9.0 and practically zero at pH 11.0.

3. There was evidence of significant inactivation of the B-P factor in the alkaline solutions.

4. The maximum adsorption of the P-P factor by fullers' earth occurred at pH 0.08, the most acid solution tested.

5. The adsorption of the P-P factor decreased gradually to a minimum at pH 6.3 and thence remained approximately constant to pH 12.07, the most alkaline solution tested.

6. Fullers' earth seemed to be a more efficient adsorbent for the B-P factor than for the P-P factor.

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THE CHEMISTRY OF JAFFE'S REACTION FOR CREATININE.

V. THE ISOLATION OF THE RED COMPOUND.*

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(Received for publication, September 21, 1928.)

In a recent number of this *Journal* (1), there was described a compound of 2 molecules of creatinine, 1 of picric acid, 3 of sodium hydroxide, and 3 of water. This was originally obtained by adding a solution of creatinine picrate in sodium hydroxide to absolute alcohol. Better yields were obtained in later experiments in which an additional molar equivalent of creatinine was used. The creatinine in this compound was found to be present in a form that does not give Jaffe's reaction. The full color value of the creatinine present could be obtained only after solution of the compound, addition of acetic acid, and heating the solution to boiling or allowing it to stand at room temperature for at least an hour.

It had previously (2) been found that, although only 1 mol of picric acid entered into the reaction, the maximum color in Jaffe's reaction was not obtained unless at least 2 mols of picric acid were present. It was, therefore, believed to be of interest to ascertain what might be precipitated by running into alcohol an alkaline mixture containing 2 mols of picric acid for each mol of creatinine. The first preparation yielded a red precipitate which, upon analysis, appeared to be a mixture of the compound previously described with a new one containing 1 molecule of creatinine, 1 of picric acid, and 2 of sodium hydroxide. By using a little more picric acid (2.5 or 3 mols) and by avoiding the use of too great an excess of sodium hydroxide, the new compound was obtained in a nearly pure condition.

*Read before the Section of Organic Chemistry, American Chemical Society, Swampscott, Mass., September, 1928.

XUM

After drying *in vacuo*, the substance forms a brilliant red, hygroscopic powder.

When dissolved in water and diluted to contain 10 mg. of creatinine per 500 cc. and compared in a colorimeter with 0.5 N $K_2Cr_2O_7$, as in Folin's original method for the determination of creatinine (3), the color obtained corresponds to only about 20 per cent creatinine instead of the calculated 26.8 per cent. But this is probably due to dissociation, for, if the solution of the new compound in 10 cc. of water is treated with a mixture of 15 cc. of

TABLE I.

Analyses of the New Compound of Creatinine, Picric Acid, and Sodium (or Lead) Hydroxide.

	Picric acid.	Nitrogen.	Sodium.
	per cent	*per cent*	*per cent*
Calculated for a compound of 1 molecule of creatinine, 1 of picric acid, 2 of NaOH....	54.2	19.9	10.9
Same with 0.5 mol H_2O..	53.1	19.5	10.7
Preparation 12..	51.6	19.6	10.8
" 13..	53.5	19.5	11.1
" 20..	52.5	20.3	11.5
			Lead.
Calculated for a compound of 1 molecule of creatinine, 1 of picric acid, 2 of lead hydroxide, 2 of H_2O..	26.6	9.76	48.1
From Preparation 13..	24.3	9.18*	51.7

* 9.15 per cent when first dissolved in dilute acetic acid; 9.20 per cent when dissolved in hot 67 per cent H_2SO_4.

1 per cent picric acid and 5 cc. of 10 per cent sodium hydroxide and is then immediately diluted to 500 cc. the full color value is obtained. It is not necessary to wait 10 minutes for the color to develop.

There can be no doubt, therefore, that it is the formation of this compound that is responsible for the red color of Jaffe's reaction. This does not altogether contradict the view previously expressed (2) that it is the formation of the red tautomer of creatinine picrate that is responsible for the production of the color, for the new compound may be regarded as a compound of the red tautomer

with 2 mols of sodium hydroxide. Treatment of fairly concentrated solutions of the new compound with hydrochloric acid results in precipitation of the red tautomer.

A solution of the new compound gives a red precipitate when treated with basic lead acetate solution. When filtered, washed, and dried over H_2SO_4, the composition of this precipitate agrees quite closely with that calculated for a compound of 1 molecule of creatinine, 1 of picric acid, 2 of lead hydroxide, and 2 of water. As in the case of the dicreatinine compounds, both the sodium and the lead compound contain more base than the formulæ would require. With the high equivalent weight of lead, this results in a decided effect on the creatinine and picric acid content.

That the nature of the combination between picric acid and creatinine in the new compounds is quite different from that in the dicreatinine compounds, is shown not only by the difference in the behavior of the creatinine but also by differences in the behavior of the picric acid. Analyses for nitrogen in the new lead compound give the same value when the substance is treated directly with hot 67 per cent sulfuric acid as when it is first treated with dilute acetic acid. Moreover, there is no marked destruction of the picric acid when a solution of the sodium compound is treated with dilute hydrochloric acid.

EXPERIMENTAL.

The analytical methods were those previously employed (1).

Preparation.

Details of Preparation 13.—To a solution of 17.2 gm. (0.075 mols) of picric acid in 300 cc. of boiling water, there were added 3.39 gm. (0.03 mols) of creatinine. After the solution had been cooled to 15°, 30 cc. of 10 per cent NaOH were added from a pipette, the mixture being stirred constantly. The red solution was decanted from the undissolved material, which was now treated with 30 cc. of 2 per cent NaOH. Complete solution was now effected, and the two solutions were mixed and allowed to stand for 15 minutes. Some sodium picrate separated. The mixture was filtered, the filtrate being received in 3000 cc. of absolute alcohol and stirred constantly. The red precipitate obtained was filtered on a

hardened paper, with suction, washed with alcohol, and dried over sulfuric acid. The yield was 3.1 gm. or 25 per cent of the calculated.

Recovery of Creatinine Picrate from the Sodium Compound.

1.49 gm. of Preparation 20 were dissolved in 35 cc. of water and the solution was than acidified with acetic acid. The precitate that formed was partly red and partly yellow. After standing overnight, it was all yellow. The crystals were filtered out and recrystallized from hot water. They melted at 222° and contained 67.4 per cent picric acid and 24.3 per cent nitrogen. The values calculated for creatinine picrate are 67.0 per cent picric acid and 24.6 per cent nitrogen. The yield was 0.89 gm. The correction for solubility (4) in the total of 85 cc. of filtrate was 0.16 gm., a total of 1.05 gm. The calculated yield was 1.18 gm.

Color Development from the New Compound.

37.5 mg. of Preparation 13 were dissolved in water, diluted to 500 cc., and compared in a colorimeter with 0.5 N $K_2Cr_2O_7$ set at 8.0 mm. The reading obtained was 11.0 mm., equivalent to 7.36 mg. of creatinine or 19.6 per cent. 37.2 mg. of the same preparation were dissolved in 10 cc. of water, a mixture of 15 cc. of 1 per cent picric acid and 5 cc. of 10 per cent NaOH was added, and the mixture at once diluted to 500 cc. The reading was 9.1 mm., equivalent to 8.92 mg. of creatinine or 26.9 per cent. This is the calculated amount.

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AN INHERITANCE STUDY OF THE DISTRIBUTION OF VITAMIN A IN MAIZE.*

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The investigations on the correlation between yellow pigmentation of certain foods and their content of vitamin A, have been reviewed adequately (1, 2). Although vitamin A is associated with yellow pigmentation in many plant and animal tissues and in many oils, yet it has been clearly demonstrated that vitamin A may be present in the absence of the yellow pigments and *vice versa*.

It is well known that the yellow dent varieties of corn (maize) contain an adequate supply of vitamin A for normal growth in the albino rat, while the white dent varieties are lacking, or at least highly deficient in this factor. This fact, together with our knowledge that the inheritance of endosperm color in maize is typically Mendelian in nature (3), provides a background for determining the mode of inheritance of this biochemical factor. Because of the rather constant difference in vitamin A content between the yellow varieties of corn and all white ones which have been investigated, it is important to study the association between yellow endosperm and vitamin A content of dent corn in relation to physiological and genetic characteristics.

Such information will be of value to the corn breeder who is crossing yellow and white dent strains of corn for the production of higher yielding strains of high feeding value and with other commercially desirable qualities. It will also be valuable to the

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farmers of some sections of the corn belt where certain locally adapted strains of white dent corn consistently give better yields than any of the yellow dent strains which have been introduced up to the present time. Where such differences exist, or when corn is being purchased for live stock feeding, some quantitative measure of vitamin A content of the grain is essential in determining the relative economy of using corn as a sole source of this factor rather than supplementing the ration with materials rich in this constituent.

Although the instances of the simultaneous occurrence of this vitamin and plant carotinoids have been generally accepted to be purely accidental, evidence of their lack of association has not been presented for corn. Comparison of the biological value of the cross white dent × yellow dent and its reciprocal, yellow dent × white dent, should provide these data. If yellow color is always associated with the higher vitamin A content in the progeny of these crosses, some chemical or physical relationship between the two may be assumed. If a genetic relationship exists under these conditions, it must be a case of close linkage of the genes responsible for these characters.

In the crosses of white dent by yellow dent, it is well known that the first hybrid generation grains appear white-capped or white at the soft starch portion of the crown with distinctly yellow horny endosperm. In the reciprocal cross, yellow by white dent, these first hybrid generation grains appear uniformly light yellow but darker than those of the preceding cross. The F_2 hybrid generation from these seeds consists of dark yellow, medium and light yellow, and pure white grains in the approximate proportion of 1 : 2 : 1. Under such conditions there is a single pair of genetic factors controlling the endosperm color relations between a white dent and yellow dent. The question arises whether or not the vitamin A content is transmitted along with yellow endosperm through the process of crossing and segregation.

Evidence for the presence of additional genetic factors for the development of yellow endosperm has been presented by East (4), Emerson (5), Anderson (6), and Hayes and Brewbaker (7). These dominant factors which lead to the development of pale or light yellow endosperm in comparison with the amber or orange-yellow which is normal to such a variety as Reid Yellow Dent, do not

appear frequently and were not encountered in the material studied.

In this study, the analysis of the vitamin A content of the corn grain in crosses of yellow and white endosperm must be considered from the view-point of a single dominant Y (yellow) and a recessive y (white) factor. Because of the process of double fertilization, there will then be four different genetic combinations possible in the endosperm tissue of the corn grains as follows:

YYY, yellow factors from both parents.
YYy, two yellow factors from female and one white from the male.
yyY, " white " " " " " yellow " " "
yyy, white factors from both parents.

An F_2 population of the cross white by yellow endosperm will consist of 25 per cent each of pure yellow and pure white endosperm grains, which may be recognized by inspection and separated from the remaining 50 per cent. The two genetic components of this light yellow class cannot be recognized accurately by inspection, and their separation was not attempted by genetic processes in the study because it was not warranted by the present degree of precision in the quantitative estimation of the vitamin A constituent.

As slight differences in degree of maturity, weathering, and other environmental agencies cause variations in the color of different ears of the same genetic constitution, it is important to reduce such variations in sampling. This was accomplished by having each color class selected in equal quantity from each F_2 ear used. As the different genotypes are distributed at random over the entire ear this procedure should insure a uniform environment for each fraction of the sample.

The material used in this study consisted of single cross hybrids and selected inbred strains of the well known varieties, Johnson County White Dent and Reid Yellow Dent. In the first set of experiments two representative samples of the open pollinated parent strains from which the inbred material and hybrids were derived, were included. The cross 461-5-1-2 (yellow dent) × 12-14-2-4 (white dent) was made reciprocally and served as foundation stock. F_2 ears were obtained from this material by hand pollination, and equal quantities of each class, yellow, light yellow,

and pure white grains, were selected from each ear to prepare the composites which were dried and ground for the biological analyses. 200 ears were used in 1926 and 100 in 1927 for these composites. The material used in 1926 came from first generation hybrid seed of the cross, yellow dent × white dent, while that used the next year came from the reciprocal cross. The purpose of this procedure was to test the possible relation of the vitamin A content to the parent variety characteristics apart from endosperm color.

TABLE I.

Composition of Rations Used to Test Various Types of Corn for Vitamin A.

Lot No.	1	2	3	4	5
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
White endosperm, parent	20				
F_2 white, yyy		20			
F_2 yellow, Yyy and YYy			20		
F_2 yellow, YYY				20	
Yellow endosperm, parent					20
Casein	15	15	15	15	15
Agar-agar	2	2	2	2	2
Lard	5	5	5	5	5
Yeast	3	3	3	3	3
Salt Mixture 185*	3	3	3	3	3
Dextrin	52	52	52	52	52

All animals were protected against rickets by irradiation with ultraviolet light.

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.

EXPERIMENTAL.

Through the breeding technique previously described, three lots of hybrid corn were secured from the same ears: (1) F_2 yellow, YYY, (2) F_2 yellow, Yyy and YYy, and (3) F_2 white, yyy. With the two parent varieties, (4) Johnson County White Dent and (5) Reid Yellow Dent, five lots of corn were used in these tests.

These experiments were conducted with albino rats selected from our stock which is a pure strain, originally secured from the Wistar Institute. Litter mates were distributed through the various lots of each series, the number and sex being shown by the

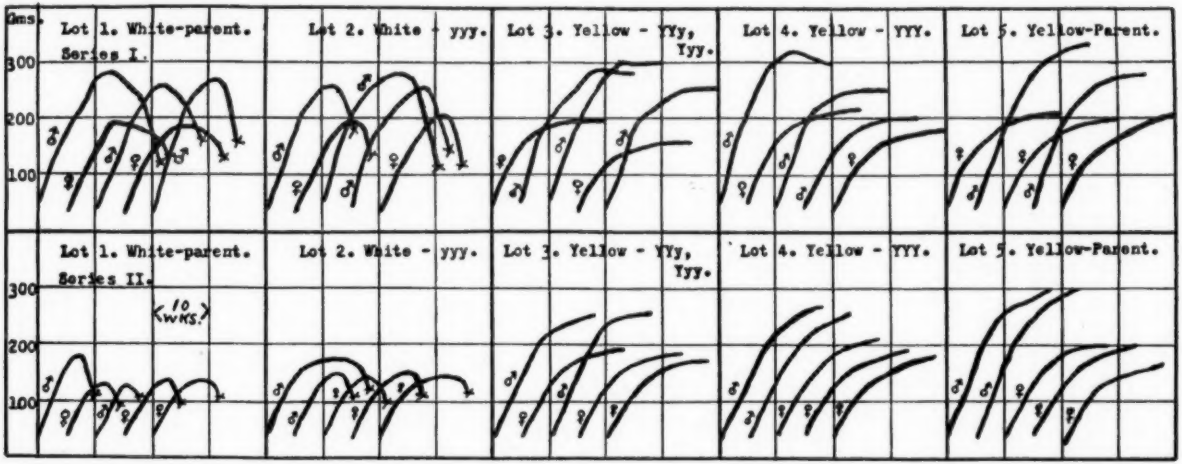

CHART 1. Showing the results of the inheritance study of vitamin A in maize. Series I, comparison of the hybrid kernels of the cross, yellow dent × white dent, with the parent strains. Series II, similar test with the reciprocal cross. Vitamin A was found to be present only in the kernels with yellow endosperms, and lacking in the kernels with white endosperms, even when the latter were grown on the same ears as those with yellow endosperms.

chart. Each rat was placed in an individual cage of a type which prevented access to excreta. The food was given *ad libitum* in the McCollum type feeding cups. The composition of the rations is given in Table I. All animals were protected against rickets by irradiation with ultra-violet light. Weekly records of weights of the animals and of food consumption were kept.

Series I (*Yellow Dent* × *White Dent*).—The corn used in these tests was produced in 1926. The results of these tests are presented in Series I of Chart 1. In comparing the Lots 2, 3, and 4, it is seen that vitamin A was present only in the kernels possessing the yellow endosperm and was lacking in the kernels of pure white endosperm, even though they were grown on the same ears as those of the yellow endosperm. The results paralleled those of the pure parent strains of white and yellow endosperm.

Series II (*White Dent* × *Yellow Dent*).—The corn used in this series was produced in 1927. The results of these tests are presented in Series II of Chart 1. It is to be noted that although the F_2 corn was produced from the reciprocal cross of that of Series I, the results were similar. The vitamin A maintained its association with only the yellow endosperm.

DISCUSSION.

In regard to the association of vitamin A in corn with the yellow pigment, Palmer (8) says: "The relation between the vitamine and color in the case of corn may be a genetic one, in which case it should be possible to transfer the vitamine to white corn." If such a transfer were possible, it would naturally be an important factor in increasing the nutritive value of some types of corn.

The results of these experiments indicate a close physiological association of vitamin A with the yellow endosperm kernel character in dent corn. It has not been possible to obtain any measurable transfer of this factor to grains possessing pure white endosperm, as rats developed ophthalmia on such corn in all cases here reported. In other tests, not reported, where rats were fed white corn segregated from the cross of yellow dent × white dent, they developed ophthalmia even when the white corn constituted 50 per cent of the ration. In these materials vitamin A was transmitted exclusively with yellow endosperm through the process of crossing and segregation.

Identical results were obtained from yellow dent crossed by white dent and likewise from the reciprocal cross. These facts indicate that any genetic relationship between these two characters must be one of very close linkage.

In these experiments, the ophthalmic reaction was used as the criterion for the presence or absence of vitamin A. All the rats on the rations containing white corn succumbed to ophthalmia, while none of those on the rations containing corn with yellow endosperm developed any symptoms of this disease. Previous to the appearance of ophthalmia, all of the rats made good growth.

At the completion of Series I, it was recognized that the relatively long period before the incidence of ophthalmia was probably due to a heavy storage of this vitamin in the animals, since they were from stock which had received a vitamin-rich ration, fortified with cod liver oil. It, therefore, seemed desirable to use rats in the next series which would have a lower storage of vitamin A. This was accomplished by placing a group of rats on a lower vitamin A diet, omitting the cod liver oil, and protecting the animals against rickets by irradiation. The rats used in Series II were selected from litters of this group. The difference in vitamin A storage of the two groups of animals used in Series I and II, undoubtedly accounts for the more rapid onset of ophthalmia in Lots 1 and 2 in Series II, which is well illustrated by the difference in the graphs of the two series.

At the 20 per cent levels of corn used in these rations, it was not possible to detect any differences in vitamin A content between the segregating genotype homozygous for yellow endosperm and the yellow class heterozygous for this kernel character. These results indicate that hybrid strains of dent corn, produced by crossing white and yellow dent corn, will possess an abundant supply of vitamin A.

SUMMARY.

1. A study was made of the association between the vitamin A content and the inheritance of the yellow endosperm kernel character in a cross of Reid Yellow Dent and Johnson County White Dent corn.

2. The vitamin A was transmitted exclusively with yellow endosperm through the process of crossing and segregation.

3. It has not been possible to obtain any measurable transfer

of vitamin A to white endosperm grains selected from the F_2 segregating ears.

4. There is a close physiological association between vitamin A and yellow endosperm kernel character in dent corn. Any genetic relationship must be one of close linkage between these two factors.

5. At the level of corn used in the rations of these experiments, the three classes possessing yellow endosperm, heterozygous F_2, homozygous F_2, and homozygous parent, were equally effective in preventing ophthalmia.

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ANIMAL CALORIMETRY.

THIRTY-SEVENTH PAPER.

THE SPECIFIC DYNAMIC ACTION OF GLYCINE GIVEN ORALLY AND INTRAVENOUSLY TO NORMAL AND TO ADRENALECTOMIZED DOGS.

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INTRODUCTION.

In a publication of 1922 Lusk (1) says: "The specific dynamic action of protein consists in a specific chemical stimulus of cellular protoplasm, which is independent of the oxidation of the material through which the stimulus is applied. It may be termed the *metabolism of amino-acid stimulation.*"

Wilhelmj and Bollman (2) have stated that the specific dynamic action of glycine amounted to more than 3 times the physiological heat value of deaminized glycine and nearly 1½ times the physiological heat value of the total amount of glycine given. The heat appearing as specific dynamic action after alanine was given was 121 per cent of the heat value of the amount of alanine deaminized. When these authors express the specific dynamic action of the

amino acid as calories for each millimol deaminized they find that the specific dynamic action of phenylalanine is twice that of alanine. In conclusion they emphasize that they do not believe, nor do they wish to imply, that specific dynamic action is always necessarily associated with deamination of the amino acid.

These investigations by Wilhelmj and Bollman seem to tally well with Lusk's view quoted above on the specific dynamic action of amino acids as dependent upon a stimulus which augments metabolism in the organism. As to how such a stimulus to increased metabolism can come about, nothing is as yet known with certainty.

In experiments on rabbits Nord (3) found that the hyperglycemia arising after injection of glycine does not occur if the adrenals have been removed. Nord (4) also found that injection of glycine into rabbits brings with it characteristic changes in the chromaffin tissue of the adrenals, indicating an increased secretion of epinephrine there. These investigations support the assumption that the injection of glycine into rabbits—subcutaneously or intravenously—results in an increased production of epinephrine.

As epinephrine has a decided capacity for augmenting metabolism, it may naturally be asked whether the adrenals have something to do with the production of the specific dynamic action of amino acids. To study this problem we have carried out investigations on dogs in order to discover whether the specific dynamic action of glycine still exists after removal of both adrenals, and whether, if such be the case, this effect is as great as in normal animals. As the experiments by Nord mentioned above were carried out by subcutaneous or intravenous administration of the amino acid, we have in our experiments supplied the amino acid not only *via* the stomach—the classical mode of procedure in experiments concerning specific dynamic action—but also by intravenous injection. That the last mentioned method of applying the amino acid would be useful for the study of the specific dynamic action was likely on the strength of the following data from the literature.

Wolf and Hele (5) obtained considerable specific dynamic action with rapid onset after intravenous injection of glycine in decerebrate dogs. Aub, Everett, and Fine (6) obtained similar results in decerebrate cats. Krzywanek (7) obtained a specific

TABLE I.
Experiments on Normal Dogs.

Calories per hr.	Increase of

TABLE I.

Experiments on Normal Dogs.

Dog No.	Date.	Experiment No.	Duration of experiment.	R.Q.	Calories per hr.		Increase of indirect calories over basal.		
					Total calculated.	Total found.	cals.	%	
	1927								
51, weight 8.7–9.5 kg.	Dec. 16	1	3 hrs.	0.96	18.55	18.42			Basal metabolism.
	" 20	3	3 " 1 min.	0.83	16.68	18.53			" "
	" 22	5	4 "	0.80	17.49	16.32			" "
	" 30	7	1 " 59.5 "	0.80	18.67	18.82			" "
	1928								
	Jan. 3	8	3 "	0.81	18.45	18.75			" "
	" 5	10	3 " 0.5 "	0.80	18.14	17.56			" "
Average				0.83	17.93	17.97			" "
51	*1927* Dec. 21	4	4 hrs. 0.5 min.	0.82	19.57	19.75	1.64	9.1	10 gm. glycine intravenously.
	1928 Jan. 4	9	4 "	0.85	20.36	20.30	2.43	13.6	10 " " orally.
56, weight 11.1–11.3 kg.	Feb. 9	1	3 hrs. 20 min.	0.82	17.43	17.24			Basal metabolism.
	" 10	2	1 " 40 "	0.88	17.50	16.38			" "
	" 14	5	1 " 40 "	0.86	16.67	15.81			" "
	" 16	7	3 " 43 "	0.92	16.92	16.96			" "
	" 23	9	1 "	0.96	17.53	19.11			" "
Average				0.89	17.17	16.95			" "
56	Feb. 10	3	3 hrs. 20.5 min.	0.881	21.00	20.35	3.83	22.3	10 gm. glycine orally.
	" 11	4	4 " 10 "	0.88	22.12	21.30	4.95	28.8	10 " " intravenously.
	" 15	6	3 " 20 "	0.92	21.19	20.02	4.02	23.4	10 " " "
	" 24	10	2 " 4 "	0.84	20.43	18.29	3.26	19.0	10 " " "
Average				0.88	21.25	19.87	4.08	23.7	

dynamic action by intravenous injection of glycine and alanine into normal dogs. In this laboratory Weiss and Rapport (8) in experiments on a dog after 10 gm. of glycine were given orally obtained a 21.4 per cent increase of the basal metabolism, subcutaneously one of 15.0 per cent, and intravenously an increase of 18.6 per cent. The work by Wilhelmj and Bollman (2) referred to above—published, however, after the conclusion of our work—also shows that the specific dynamic action of amino acids makes itself felt after intravenous injection in the dog. Contrary to all these investigators Liebeschütz-Plaut and Schadow (9) have not found any specific dynamic action in the anesthetized dog after intravenous injection of glycine or other amino acids.

Experimental Procedure.

The experiments have been carried out with the calorimeter long employed in this laboratory. As experimental animals we have used bitches which prior to the experiments have been trained to lie quietly in the calorimeter. Any movements are detected by a registering device. In case the animal moves sufficiently to influence the metabolism to any appreciable extent, the results are discarded. For some time before the commencement of the experiments and all the time while they lasted, the normal dogs received daily a standard diet. Every afternoon at 5 they received the same diet that has long been used in this laboratory for dogs of similar size (10): 100 gm. of lean beef heart, 100 gm. of biscuit meal, 20 gm. of lard, and 10 gm. of bone ash. The experiments as a rule were commenced 17 to 18 hours after the meal. However, after double adrenalectomy, the animals in some cases refused to take food, which accounts for the low R.Q. in the basal determinations. While this state of undernutrition may somewhat lower the basal metabolism, it would not affect the response to glycine.

The calculations of the experiments have been done in the way usually practised in this laboratory.

All injections have been carried out with due attention to aseptic precautions.

EXPERIMENTAL.

The intention was first to investigate the specific dynamic action following oral and intravenous administration of glycine to the nor-

mal animal, and later to repeat the same experiment upon the same animal after the removal of the adrenals. Those animals, however, on which such preliminary tests were carried out, unfortunately died so soon after the removal of the second adrenal that the time at our disposal did not allow any further attempts of this nature. As these preliminary experiments had occupied so much time, we were obliged to proceed directly with extirpation of the adrenals without having examined the specific dynamic action before the operation.

A. Comparison between Oral and Intravenous Administration of Glycine to Normal Dogs.

In Table I will be found a tabulation of the experiments on Dog 51. Six experiments gave an average basal metabolism of 17.93 calories per hour. Intravenous injection of 10 gm. of glycine resulted in an average augmentation of the metabolism by 9.1 per cent; oral administration of the same quantity of glycine gave an increase of 13.6 per cent. In Dog 56 (Table I) five experiments gave an average basal metabolism of 17.17 calories. Intravenous injections of 10 gm. of glycine into this animal resulted in an increase of 28.8, 23.4, and 19.0 per cent respectively or an average of 23.7 per cent, and on oral administration an increase of 22.3 per cent was obtained. In these two animals, therefore, four experiments in all were carried out with intravenous injection of glycine, all of them showing an undoubted specific dynamic action. In Dog 51 somewhat less specific dynamic action was obtained on intravenous injection than on oral administration. Dog 56, on the other hand, reacted with a more marked increase of the metabolism on intravenous injection than on oral administration of glycine. In all three experiments on this animal a somewhat more marked specific dynamic action followed after intravenous injection than was obtained by Weiss and Rapport (8) in similar experiments in this laboratory with the same calorimeter. The average increase in our experiments and in those of Weiss and Rapport (see their Table IX) is: orally 19.1 and intravenously 19.8 per cent. Corresponding figures for the absolute increase were: orally 3.26, intravenously 3.39 calories. These figures are so much alike that the difference between them lies within the limits of variability. It would seem, therefore, as if *intravenous and oral administration of glycine in the dog resulted in equally marked increases of metabolism.*

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B. Experiments on Adrenalectomized Dogs.

The adrenals were extirpated under ether anesthesia with due attention to usual aseptic precautions. In order to diminish the effect of operative shock the adrenals were removed at two sittings at intervals of about 7 days. The right adrenal, being surgically the more difficult, was removed at the first operation, the left at the second. The metabolic tests were commenced on the following day. As the basal metabolism might conceivably alter from day to day after adrenalectomy, it was determined every morning before the glycine experiment was carried out. The value obtained for the basal metabolism the same morning was thus taken as the starting point for the calculation of the increased effect obtained after administration of glycine on the same day. The animals were in good condition at the time of the experiments. Dog 53 died 3 days after the last experiment. No trace of the adrenal could be found at the autopsy. Dog 55 died a couple of days after the test. No postmortem examination was made. Dog 57 was still in excellent condition 43 days after removal of the second adrenal. There was no doubt whatever that both adrenals had been completely removed and that no accessory adrenals could be observed at the operation in spite of careful search. Nevertheless exploratory laparotomy was performed on the 43rd day for further search for any remnant of the adrenals proper or accessory adrenals. No trace of adrenal tissue could be found. Dr. S. F. Cooper who followed the further fate of the dog gives the following particulars: "I killed the dog on June 5, 80 days after removal of the second adrenal. Careful postmortem examination showed no signs of adrenal tissue. Since nothing suspicious was seen no microscopic sections were made. At the time of death the dog was a little weak and quite emaciated. It had a very good appetite, however, and the indications were that it would have lived indefinitely."

A glance at Table II shows that in all the experiments on the adrenalectomized dogs we obtained an increase of the metabolism after administering 10 gm. of glycine either orally or intravenously. The average increase orally was 10.2 per cent, intravenously 12.3 per cent. Corresponding figures in the normal animals were 19.1 and 19.8 per cent. The figures for the basal metabolism, however, were very much higher in the operated animals of the same size,

TABLE II.
Experiments on Adrenalectomized Dogs.

			Calories per hr.	Increase of

TABLE II.

Experiments on Adrenalectomized Dogs.

Dog No.	Date.	Experiment No.	Duration of experiment.	R.Q.	Calories per hr.		Increase of indirect calories over basal.		
					Total calculated.	Total found.			
	1928						*cals.*	*%*	
53, weight	Jan. 9								Right adrenal removed.
14.1–13.7	" 17								Left " "
kg.	" 18	2	2 hrs. 0.5 min.	0.76	24.06				Basal metabolism.
	" 18	3	2 " 40 "	0.79	27.09		3.03	12.6	10 gm. glycine orally.
	" 19	4	1 " 20 "	0.75	23.35				Basal metabolism.
	" 19	5	2 " 40 "	0.77	26.67		3.32	14.2	10 gm. glycine intravenously.
	" 20	6	2 "	0.74	22.94				Basal metabolism.
	" 20	7	2 " 40 "	0.79	24.13		1.19	5.2	10 gm. glycine orally.
	" 23								Found dead. Autopsy, no trace of adrenals.
55, weight	Jan. 23								Right adrenal removed.
11.9 kg.	Feb. 27								Left " "
	" 28	9	2 hrs. 40 min.	0.85	23.66	21.68			Basal metabolism.
	" 28	10	2 " 39.5 "	0.83	26.07	27.84	2.41	10.4	10 gm. glycine intravenously.
57, weight	Mar. 1								Right adrenal removed.
14.2–14.0	" 7								Left " "
kg.	" 9	3	1 hrs. 10 min.	0.77	30.12	31.60			Basal metabolism.
	" 9	4	2 " 43 "	0.78	34.47	33.58	4.35	14.4	10 gm. glycine orally.
	" 10	5	1 " 20 "	0.69(?)	34.97				Basal metabolism.
	" 10	6	2 "	0.72	38.35		3.38	9.7	10 gm. glycine orally.
	Apr. 19								Exploratory laparotomy, no trace of adrenals.
	June 5								Killed. Autopsy, no trace of adrenals.

and on this ground the percentage figures in these are not directly comparable with those in the normal animals. It would seem more correct to compare the figures of the absolute increase per hour. These figures in the adrenalectomized animals were on an average: orally 2.99 and intravenously 2.87 calories. These figures are more like those obtained in normal animals, which were 3.26 and 3.39 calories. The differences between the figures for normal and adrenalectomized animals would not seem to be sufficiently great to lie outside the limits of variability, which is

TABLE III.

Alcohol Checks.

Date.	Experiment No.	Duration of experiment.	R.Q.	Calories.	
				Indirect.	Direct.
1927		*hrs.*			
Nov. 29	248	5	0 666	25.56	26.70
" 30	249	3	0.669	28.46	28.91
Dec. 1	250	5	0.661	25.12	26.59
1928					
Jan. 6	251	3	0.664	14.28	14.42
Feb. 17	252	4	0.666	38.71	37.47
" 22	254	2	0.667	33.95	35.90
Mar. 13	256	3	0.665	24.48	24.92
Average.....			0.665	27.22	27.85

During Experiment 253 on February 21 leaks on the registering tambour outside the calorimeter and in the rubber tubes in the bed were discovered. The R.Q. of Experiment 255, March 12, was 0.647 and is not included in the averages given above.

rendered more apparent by comparing Table I with Table II. Even should the experiments on the adrenalectomized animals have shown a lower specific dynamic action, this could have been explained as due solely to another circumstance, for Wilhelmj and Bollman (2) emphasize that the specific dynamic action can, as it were, be masked in cases when the basal metabolism shows an abnormally high figure for the size of the animal.

It has been shown by the experiments, therefore, *that even in dogs deprived of their adrenals glycine exerts a specific dynamic action after oral as well as intravenous administration and that this effect,*

reckoned as an absolute increase in the number of calories, is very nearly as great as in normal dogs. It would seem clear from this, then, that the adrenals do not play any significant part in the production of the specific dynamic action of glycine.

The basal metabolism of the adrenalectomized Dog 55 was 35 per cent higher than that of the normal Dog 56 which had about the same weight. Adrenalectomized Dog 57, which weighed 14 kilos, had a basal metabolism of 34.5 calories per hour, which contrasts with one of 22 calories per hour usually obtained in a dog of that size. The increase exceeds 50 per cent. This contrasts sharply with the work of Aub, Forman, and Bright (11) who found in cats that adrenalectomy markedly reduced the metabolism.

C. Alcohol Checks.

For the purpose of checking the calorimetric determination in the course of the experiments control tests have been made from time to time by burning an alcohol lamp in the calorimeter and thereby determining the combustion quotient, *etc.* The heat production has been calculated from the CO_2 production. See Table III.

SUMMARY.

In all four experiments with intravenous injection of 10 gm. of glycine, carried out on normal dogs, a clear specific dynamic action was obtained. The values of this effect average about the same as by oral administration of the same quantity of glycine.

Also in all the experiments on adrenalectomized dogs undoubted specific dynamic action was obtained after 10 gm. of glycine were given orally or intravenously. The average increase per hour of the number of calories was very nearly the same as in normal dogs: 2.99 calories orally and 2.87 intravenously, as against 3.26 and 3.39 respectively in normal animals.

It would seem clear therefore that the adrenals do not play any significant part in the production of the specific dynamic action of glycine.

After adrenalectomy the basal metabolism of the dogs was markedly increased.

We would like to express our thanks to Dr. J. E. Sweet in whose laboratories the adrenalectomies were carried out.

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DIFFERENCES IN SERUM AND PLASMA CONTENT OF CHOLESTEROL ESTER.

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In conducting cholesterol ester determinations in parallel on the serum and plasma of blood from the same animal, discrepancies have been observed. The cholesterol ester content of serum

TABLE I.

Animal No.	Serum.		Sodium citrate plasma.		Potassium oxalate plasma.		Heparin plasma.	
	Total cholesterol.	Ester cholesterol.	Total cholesterol.	Ester cholesterol.	Total cholesterol.	Ester cholesterol.	Total cholesterol.	Ester cholesterol.
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
1279	208	144	206	128				
1566	140	104	139	93				
5284	110	77	108	68				
13	152	87	150	55				
1414	195	119					194	119
1462	188	134					189	135
H 8	177	117			175	93	170	114
J 55	203	134			201	119	198	132
5082	197	151			185	128	185	156

was invariably greater than that of plasma while the total cholesterol values showed no differences. Since sodium citrate, 4 mg. per 1 cc. of blood, was being used as the anticoagulant in the blood from which the plasma was taken, other plasma samples were investigated in which potassium oxalate, 2 mg. per 1 cc. of blood, or heparin, 1 mg. per 5 cc. of blood, were used. The results showing the differences observed are given in Table I. All determinations were done on normal cow blood using Bloor's (1)

method for total cholesterol determinations and Bloor and Knudson's (2) for cholesterol ester determinations. All blood samples from an individual animal were drawn in succession from the jugular vein.

The data presented indicate that plasma, obtained from blood in which either sodium citrate or potassium oxalate have been used as anticoagulants, contains less cholesterol bound as ester than does serum from blood drawn from the same animal and at the same time. When heparin was used as the anticoagulant no difference in cholesterol ester content was observed between serum and heparin plasma. Although the differences found to exist between the cholesterol ester content of serum and either citrate or oxalate plasma are not very great, they are, nevertheless, of importance in that they are a source of potential error. Obviously the cholesterol ester values obtained for serum or heparin plasma more nearly represent the correct values for the non-cellular portion of the blood than do the values obtained for citrate or oxalate plasma since either sodium citrate or potassium oxalate appear to be able to cause a partial hydrolysis of that portion of the serum cholesterol generally determined as ester cholesterol.

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CHOLESTEROL ESTERASE IN ANIMAL TISSUES.

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INTRODUCTION.

From time to time evidence has been obtained in this laboratory indicating the presence in animal tissues of some very active cholesterol ester-splitting substance. The first instance of this kind was in the case of a young bull experimentally infected with *Bacillus tuberculosis*. 10 days prior to the death of this animal its total serum cholesterol was 90 mg. per cent and the serum ester cholesterol was 58 mg. per cent. As another cholesterol and cholesterol ester reading was desired, blood was taken from the heart at the time of autopsy, 2 days after the animal's death. At this time the total serum cholesterol was 69 mg. per cent while the serum ester cholesterol was 0. Other similar instances may be cited. A calf dead of an intestinal infection was autopsied 24 hours following death and blood taken from the heart at autopsy. Serum from this blood contained 144 mg. per cent total cholesterol but none was bound as ester. (Normal bovine blood serum usually contains from 50 to 70 per cent of the total cholesterol bound with fatty acid as ester.) A rabbit dying of a broken neck was bled 5 minutes post mortem. Its total serum cholesterol was 31 mg. per cent while the serum ester cholesterol was 0. 2 days earlier its total serum cholesterol had been 38 mg. per cent and the serum ester cholesterol was 23 mg. per cent. The complete disappearance of cholesterol ester from blood serum taken from an animal after death is not always observed but in the instances in which it is not observed a marked diminution is found when compared with the most recent determination made during the life of the animal.

The nature of these findings suggested two possible explanations. The first of these was that saturation of the blood with carbon dioxide following death might cause a splitting of cholesterol ester into free cholesterol and fatty acid. The second was that the hydrolysis was due to enzyme action.

Mueller (1) in autolysis experiments with blood and mixtures of blood and liver was unable to detect any evidence of hydrolysis of cholesterol esters indicating the presence of cholesterol ester-splitting enzymes and he disputed the earlier positive results of Shultz (2) and Cytronberg (3). Porter (4) in working with esterases extracted from various animal tissues obtained evidence indicating that cholesterol esterase was present in at least a few tissues although the degree of activity was slight in most instances where it was found to be present at all. The one exception to this was in a preparation from human skin which showed quite marked cholesterol ester-splitting ability. Nomura (5) using extracts of dog, cow, horse, swine, and rabbit tissues observed an enzyme, capable of splitting synthetic cholesterol oleate, present in liver, pancreas, intestinal and gastric mucosa, spleen, kidney, and muscle but not in blood.

EXPERIMENTAL.

The possibility that the splitting of serum cholesterol might be due to the saturation of the blood with carbon dioxide following death was considered first.

Several samples of cow blood were drawn, in duplicate, heparin being used as the anticoagulant. Carbon dioxide was allowed to bubble slowly through one sample from each animal for half an hour. The other samples were allowed to stand at room temperature. All were then centrifuged and total and ester cholesterol determinations made on the sera. The results were entirely negative and no evidence was obtained that carbon dioxide caused any change in the serum cholesterol ester content.

The second possibility, that of enzyme action, was considered in some detail and the results obtained are given in accompanying tables.

The experimental procedure was usually as follows: Guinea pig tissues were, with one exception, used as the source of the esterase. Each tissue under test was taken from a freshly killed animal, weighed, and ground in a mortar, 5 cc. of physiological salt solution per gm. of tissue being gradually added. The mixture was then filtered through paper. The extract prepared

in this way was found to contain no cholesterol. The esterase was tested against the cholesterol ester in normal cow serum. 2 cc. of salt solution tissue extract were added to 3 cc. of cow serum in a test-tube, mixed well, and incubated at 37° for 3 hours, no preservative being used. Control tubes contained 2 cc. of physiological salt solution and 3 cc. of cow serum. One experiment was conducted in which a water suspension of cholesterol oleate was used instead of cow serum. After 3 hours incubation the total cholesterol was determined by the method of Bloor (6) and

TABLE I.

Tissue extract.	Guinea pig tissues and cow serum.				Rabbit tissues and cow serum.		Guinea pig tissues and water suspension of cholesterol oleate.	
	Experiment 1. (Incubated 3 hrs.)		Experiment 2. (Incubated 24 hrs.)		Experiment 3. (Incubated 3 hrs.)		Experiment 4. (Incubated 3 hrs.)	
	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Control	175	127	156	79	205	170	66	61
Liver	174	84	159	55	198	102	72	46
Kidney	177	97	157	42	201	107	70	46
Muscle	175	98	156	54	203	117	67	52
Lung	177	112	153	36				
Brain	175	95	158	37				
Spleen	175	110	156	54				
Heart	174	114	156	54				
Testicle	174	108	154	72				

cholesterol ester by the method of Bloor and Knudson (7). The results obtained are summarized in Table I.

The exact temperature at which the cholesterol ester-splitting activity is destroyed was not determined. Boiling the salt solution tissue extract for 5 minutes completely destroyed its ability to split cholesterol ester. The temperature of inactivation lies somewhere below 67° as heating a salt solution extract of guinea pig liver at 67° for 30 minutes yielded the results given in Table II.

The degree of hydrolysis of serum cholesterol ester attained is influenced by the time that the reaction is allowed to progress and

by the concentration of the salt solution tissue extract as shown by the data in Table III. In this experiment an extract of guinea pig liver and normal cow serum were used. In one series the extract was used undiluted as in previous experiments, while in the other series it was diluted 1:4 with salt solution. Samples in each series were incubated for periods of 1, 3, 6, and 24 hours at 37°. 2 cc. of tissue extract and 3 cc. of cow serum were used as before. The control tube was incubated for 24 hours.

TABLE II.

Guinea Pig Liver and Cow Serum. Incubated at 37° for 24 Hours.

Tissue extract.	Total cholesterol.	Cholesterol ester.
	mg. per cent	*mg. per cent*
Control	167	133
Liver	167	97
Liver heated to 67° for 30 min.	168	132

TABLE III.

Guinea Pig Liver and Cow Serum.

Time of reaction.	Undiluted liver extract.		Liver extract diluted 1:4.	
	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.
hrs.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	165	116	167	119
3	168	107	167	114
6	168	104	167	105
24	167	97	168	99
Control (2 cc. salt solution and 3 cc. cow serum).	167	133		

DISCUSSION.

The results obtained are in very close agreement with those reported previously by Nomura and are published confirmatory to those observations.

By incubating salt solution extracts of various animal tissues with blood serum or suspensions of cholesterol oleate a decrease

in the cholesterol ester content of the sera or suspensions could be demonstrated with no accompanying change in the total cholesterol. This reaction appeared to be progressive with time and dependent upon the concentration of the tissue extract. Heating the tissue extract to 67° destroyed its ability to split cholesterol ester. The ester hydrolysis is thus probably due to an enzyme that is universally present in animal tissues. All attempts to make the reaction progress to completion, as it apparently sometimes does in the animal body following death, were unsuccessful.

The fact that cholesterol esterase is present in all tissues argues that it has an important and wide-spread function. In view of the complete absence of knowledge concerning the function of cholesterol itself, surmises with regard to the nature of the rôle that cholesterol esterase plays in the animal organism would be unfruitful at this time.

Free cholesterol and cholesterol ester maintain a very constant relationship in the blood serum of a given animal over a period of time and Mueller (8) has shown that the proportion of free to combined cholesterol in the blood serum is approximately the same as that existing in the chyle during the absorption of either free cholesterol or cholesterol ester from the gastrointestinal tract. The fact that during the life of an animal a ratio between free and combined cholesterol is maintained, while soon after death the cholesterol bound as ester decreases, would indicate that the breakdown in the mechanism controlling this relationship was unequal. That is, cholesterol ester formation ceases while hydrolysis of cholesterol ester by an esterase still continues.

SUMMARY.

1. Blood serum obtained from an animal post mortem always contains less cholesterol ester than did serum obtained from the same animal preceding death and often contains none.

2. Passage of carbon dioxide into freshly drawn blood has no effect on the cholesterol ester content of its serum.

3. Cholesterol esterase capable of splitting either cholesterol ester in normal cow serum or cholesterol oleate in water suspension is present in many animal tissues.

4. The continued activity of cholesterol esterase post mortem

with cessation either of cholesterol ester formation or absorption may explain the complete absence or diminution of cholesterol ester in blood serum obtained from an animal after death.

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THE HYPERCHOLESTEROLEMIA OF FASTING AS INFLUENCED BY THE SEPARATE ADMINISTRATION OF FATS, CARBOHYDRATES, AND PROTEINS.

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INTRODUCTION.

In a previous publication (1) it was demonstrated that the high serum content of cholesterol, associated with fasting, could be rather promptly lowered by the feeding of a mixed meal to the fasting animal. At the time, the suggestion was made that the rise in serum cholesterol observed during periods of fasting might be related to the increased utilization of body fat taking place at that time and the decrease following feeding might accordingly be due to the prompt action of administered food in sparing body fat.

It seemed proper to infer that if the changes in serum cholesterol during and after fasting were truly related to some special functional connection between cholesterol and fat metabolism this would also be reflected in some difference in the influence of feeding with the separate constituents, fat, carbohydrate, or protein, to the fasting animal. This view has been subjected to the test of experiment.

A number of investigators have maintained, on the basis of indirect experimental evidence, that cholesterol has some function in fat metabolism. The data for or against this assumption may be briefly outlined.

Mayer and Schaeffer (2) found that within an animal species for any organ the proportionate content of fatty acid and cholesterol was quite constant. They termed this proportion of fatty acid to cholesterol the "lipocytic coefficient." Terroine and Weill (3) found that inanition caused wide variations in the lipoid index of muscle but had little or no effect on that of the parenchymatous organs. Ingestion of food caused a transitory

change in the lipoid index of the liver without exerting any influence on that of other tissues. The constancy of the relationship of fats to cholesterol suggested that they were in a combination characteristic of the tissue. Terroine (4) observed that the ratio $\frac{\text{cholesterol}}{\text{total fatty acids}}$ in dog blood was a constant for the same animal at different times and very near a constant for the species. However, he noted (5) that during fasting the constant decreased irregularly. Mayer and Schaeffer (6) found that during absolute fasting the ratio of cholesterol to fatty acids of all tissues examined, increased. Morita (7) confirmed this observation. Hueck and Wacher (8) believed that their experiments indicated that cholesterol had a prominent rôle in intermediate fat metabolism. They observed that cholesterol added to food appeared in the blood stream combined as esters with fatty acids, and also that the artificial enrichment of the diet in cholesterol not only led to a hypercholesterolemia but to an increase in the fatty acid fraction of the blood lipoids. Bloor (9) noted a relationship between fat, lecithin, and cholesterol and also between cholesterol and its esters in normal blood that was constant within narrow limits for the individual and within wider limits for the species. Anything tending to result in a disturbance of this equilibrium was followed by an attempt at restoration either by elimination or by an establishment of the relationship again on a higher or lower level. Cholesterol was found to be the most constant and least easily disturbed of the lipoid constituents of normal blood. Bloor has repeatedly suggested that cholesterol has some important function in late fat metabolism (10–12). These suggestions have been based largely on the relationship found to exist between blood fat, lecithin, and cholesterol in normal and pathological human blood. Oser and Karr (13), from data obtained on normal human blood, failed to note a constant cholesterol-lecithin ratio. Terroine, Bonnet, Kopp, and Vechot (14) were unable to demonstrate any constant relationship between the aliphatic acid and sterol content of various fatty seeds or microorganisms naturally rich in fat. They did, however, obtain evidence that, in plants and microorganisms, sterols were formed at the expense of the fats. Artom (15), in a comparative study of the variation of the fatty acids and cholesterol content during artificial circulation or autolysis of the liver of a normal dog, was not able to formulate any hypothesis as to the relation between cholesterol and the fats or their cleavage products.

Experimental evidence concerning the effect of ingestion of fat upon the cholesterol content of the blood is conflicting. Beumer (16) found that during digestive lipemia an increase in serum cholesterol occurred. This increase failed to take place in a dog with a biliary fistula indicating that the increase in serum cholesterol during lipemia probably originated in the bile. Bloor (17) was unable to demonstrate any definite changes in blood cholesterol following fat ingestion. Knudson (18) fed dogs olive oil and found no constant changes in their blood cholesterol content during the resulting lipemia. He did, however, find that an increase in the ester fraction of the blood cholesterol occurred and this increase was most

marked in the corpuscles, which normally are considered to contain no ester cholesterol. Chauffard, Laroche, and Grigaut (19) found that the increase in the cholesterol content of the blood observed by them after a meal containing fat was far beyond the amount of cholesterol ingested with the food. Leites (20) fed dogs olive oil and during the resulting hyperlipemia observed an increase in the blood cholesterol, largely as free cholesterol.

TABLE I.

Effect on Fasting Hypercholesterolemia of Feeding with Pure Carbohydrate.

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	*mg. per cent*	*per cent*
Rabbit 13	Before fasting.	58	26	45
	After 48 hrs. without food.	105	67	64
	3 hrs. after 8 gm. glucose.	90	52	58
Rabbit 14	Before fasting.	59	24	41
	After 48 hrs. without food.	78	43	55
	3 hrs. after 8 gm. glucose.	61	26	43
Rabbit 8	Before fasting.	54		
	After 48 hrs. without food.	83		
	3 hrs. after 10 gm. cane sugar.	71		
Rabbit 17	Before fasting.	41		
	After 48 hrs. without food.	49		
	3 hrs. after 10 gm. cane sugar.	39		
Swine 390	Before fasting.	194	117	60
	After 67 hrs. without food.	240	160	67
	2 hrs. after 200 gm. glucose.	214	119	56
	6 hrs. after 200 gm. glucose.	201	117	58

EXPERIMENTAL.

The general experimental plan was to fast animals until a definite increase in serum cholesterol content had been established. The fasting period was 48 hours in the case of the rabbits used and 67 hours in the case of the swine. To terminate the fast the animals were fed with either pure carbohydrates (glucose and cane sugar), proteins (gelatin and casein), or fats (olive oil and butter fat). The rabbits had to be fed by stomach tube but this was not necessary in the case of the swine. Blood samples were taken at the

beginning of the period of fasting, just before feeding at the termination of the fast, and at 3 to 3½ hours following feeding in the case of the rabbits and 2 and 6 hours in the case of the swine. Blood from the rabbits was obtained from the marginal ear vein and that from the swine was drawn from the tail. Bloor's (21) method was used for the total serum cholesterol determinations

TABLE II.

Effect on Fasting Hypercholesterolemia of Feeding with Pure Protein.

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	*mg. per cent*	*per cent*
Rabbit 12	Before fasting.	59	21	36
	After 48 hrs. without food.	86	45	52
	3 hrs. after 8 gm. gelatin.	87	45	52
Rabbit 15	Before fasting.	70	33	47
	After 48 hrs. without food.	121	63	52
	3 hrs. after 8 gm. gelatin.	124	64	52
Rabbit 66	Before fasting.	27		
	After 48 hrs. without food.	49		
	3 hrs. after 8 gm. gelatin.	54		
Rabbit 67	Before fasting.	36		
	After 48 hrs. without food.	56		
	3 hrs. after 8 gm. gelatin.	66		
Swine 392	Before fasting.	142	65	46
	After 67 hrs. without food.	164	100	61
	2 hrs. after 200 gm. casein.	165	103	62
	6 hrs. after 200 gm. casein.	145	76	52

and Bloor and Knudson's (22) for the cholesterol ester determinations. The results are recorded in Tables I to III.

To test further any demonstrable alteration in serum cholesterol content resulting from or accompanying an increase in fat metabolism, four rabbits were given slightly more than their caloric requirements of olive oil for a period of 2 days. They were bled at the beginning of the experiment and at the end of the 1st and 2nd days. The results obtained are given in Table IV.

DISCUSSION.

The separate administration of either carbohydrate, protein, or fat to an animal, deprived of food for a sufficient period of time to produce a fasting hypercholesterolemia, resulted in all cases in a decrease in the serum cholesterol and cholesterol ester content. Carbohydrate produced perhaps the most marked and rapid

TABLE III.

Effect on Fasting Hypercholesterolemia of Feeding with Pure Fat.

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	*mg. per cent*	*per cent*
Rabbit 18	Before fasting.	67	37	55
	After 48 hrs. without food.	86	60	70
	3½ hrs. after 5 cc. olive oil.	77	44	57
Rabbit 19	Before fasting.	50	20	40
	After 48 hrs. without food.	83	50	60
	3½ hrs. after 5 cc. olive oil.	76	46	61
Rabbit 4	Before fasting.	30		
	After 48 hrs. without food.	56		
	3½ hrs. after 5 cc. butter fat.	47		
Rabbit 62 A	Before fasting.	76		
	After 48 hrs. without food.	111		
	3½ hrs. after 5 cc. butter fat.	100		
Swine 391	Before fasting.	130	51	39
	After 67 hrs. without food.	167	97	58
	2 hrs. after 100 gm. butter fat.	154	91	59
	6 hrs. after 100 gm. butter fat.	146	86	59

decrease, due probably to the fact that it was most available to the animal in the fasting condition. There was a very definite latent period following the feeding of protein during which the serum cholesterol either remained constant or rose slightly but the observation on the swine would indicate that this was no more than transient and perhaps represented the period of time necessary for the conversion of protein into a more readily metabolizable substance. Feeding with fat produced a decrease in serum cholesterol and cholesterol ester that was only slightly less marked,

as to rate and extent, than when carbohydrate had been fed. Thus these experiments, so far as they went, failed to demonstrate that the cholesterolemia of fasting could be ascribed to any disproportion in the type of food material being used by the animal at this time as a source of energy. That is, no evidence was obtained to indicate that the fasting hypercholesterolemia was related to the increased utilization of fat taking place at this time

TABLE IV.

Effect on Serum Cholesterol Level of Feeding Pure Fat to Rabbits (Olive Oil).

Time of experiment.	Manipulation.	Rabbit 6.			Rabbit 16.			Rabbit 58.			Rabbit 62B.		
		Total serum cholesterol.	Serum cholesterol ester.		Total serum cholesterol.	Serum cholesterol ester.		Total serum cholesterol.	Serum cholesterol ester.		Total serum cholesterol.	Serum cholesterol ester.	
hrs.		*mg. per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*per cent*
0	Bled 8 cc. All food removed from cages.	27	7	26	59	19	32	52	17	33	39	13	33
½	20 cc. olive oil.												
22	20 cc. olive oil.												
24½	Bled 8 cc.	38	10	26	58	21	36	55	18	33	46	10	22
46	20 cc. olive oil.												
48½	Bled 8 cc.	42	10	24	56	24	44	51	19	37	42	13	31

since feeding with pure fat resulted in a lowering of the serum cholesterol level in much the same fashion as did feeding with carbohydrate, or protein, or a mixed meal. In like manner it was made quite evident that the hypercholesterolemia of fasting was related in no selective way to the metabolism of either carbohydrate or protein.

In view of the amount of indirect evidence indicating some relationship between cholesterol and fat metabolism another type

of experiment was conducted, the effect of fasting upon the blood serum cholesterol content being borne in mind. Four rabbits were maintained for 48 hours on a diet of fat alone. Three of these animals failed completely to show any significant increase in serum cholesterol content while one showed a moderate rise. Observations on other rabbits, fasted for a period of 48 hours, demonstrated that, with no food at all, a very definite increase occurs. It therefore seems evident that the hypercholesterolemia of fasting is not dependent upon the increase in fat metabolism taking place at this time and thus that cholesterol does not have a function in fat metabolism under the conditions of these experiments. If the increased serum cholesterol content of fasting could possibly be considered as bearing a relationship to the increased metabolism of fat taking place at that time then animals fasted except for their caloric requirement in fat should have been expected to show at least the degree of hypercholesterolemia that rabbits fasted for a corresponding period of time would have shown. Such was not the case for, in fact, maintenance for 48 hours on fat alone resulted in no change in serum cholesterol in any except one rabbit. This one animal showed some increase which, however, might very easily be accounted for, in the light of three negative animals, as being due to failure to utilize the fat fed with a resultant fasting hypercholesterolemia.

SUMMARY AND CONCLUSIONS.

1. The separate administration of either fat, carbohydrate, or protein to a fasting animal causes a decrease in the hypercholesterolemia similar to that observed following feeding with a mixed meal.

2. A diet of fat alone for 48 hours causes no significant increase in serum cholesterol.

3. The experimental evidence presented adds no weight to the assumption so often made in the past that cholesterol has a function in fat metabolism.

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THE EFFECT OF AGE ON THE TOTAL AND COMBINED CHOLESTEROL OF THE BLOOD SERUM.

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INTRODUCTION.

The fact that the physiological function of cholesterol and its esters has not yet been established makes it highly important to utilize every available opportunity to study its variations in the hope of obtaining evidence, which, when added to what is already known, may yield a clearer and more complete picture as to the actual rôle of cholesterol in the physiology of the animal organism. A study of the changes, in both total and combined cholesterol of the blood serum, as related to age, seemed of importance for two reasons. In the first place, it offered an opportunity to observe its variations under conditions, so far, not thoroughly studied. In the second place, in a great deal of work already in the literature, on variations in blood cholesterol as related to various disease processes or physiological phenomena, the factor of age would have vitiated the results had it been considered. It is, therefore, of importance to determine to what extent age does actually influence the cholesterol and cholesterol ester level of the blood serum.

A number of investigators have recognized that differences in blood cholesterol content occurred in organisms of differing ages or stages of development. Slemons and Curtis (1) observed that fetal blood contained an amount of cholesterol approximately equal to the free cholesterol of the maternal blood and it was present entirely in the uncombined state. Chauffard, Laroche, and Grigaut (2) found that blood from the umbilical vein, obtained from infants immediately after birth, contained very much less cholesterol than the maternal blood. The average for four cases was found by these investigators to be 55 mg. per cent. Banu, Negresco, and Heresco (3) noted that the cholesterol content of infants' serum increased from an

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average of 47 mg. per cent at the age of 1 to 2 weeks to 55 mg. per cent at the age of 8 to 10 months, both values being extremely low when compared with similar values for adult serum. György (4) observed that funicular serum contained 54 to 83 mg. per cent cholesterol whereas the maternal serum contained from 220 to 279 mg. per cent. Hellmuth (5) confirmed the observations of previous investigators concerning the low cholesterol content of new born infant blood serum but, contrary to the observation of

TABLE I.

Calves That Had Not Suckled Dam at Time of First Bleeding.

Animal No.	Age.	Total serum cholesterol.	Combined serum cholesterol.	
		mg. per cent	*mg. per cent*	*per cent*
130, Holstein (female).	1 hr.	22	0	0
	48 hrs.	44	17	38
	8 days.	87	38	43
	21 "	153	69	45
	35 "	113	71	63
	57 "	112	68	61
	71 "	157	123	78
	99 "	141	116	82
	148 "	127	88	69
	199 "	93	69	74
132, Holstein (female.)	1 hr.	27	0	0
	48 hrs.	48	18	38
	8 days.	102	44	43
	21 "	165	90	54
	35 "	126	76	61
	57 "	94	61	65
	71 "	122	99	82
	99 "	130	100	77
	148 "	114	84	73
	199 "	91	66	72

Slemons and Curtis, failed to find the total amount of cholesterol present in the free form. Parhon and Parhon (6) found that in extremely old people (over 70 years of age) a definite hypercholesterolemia existed. Roffo (7) observed that in rats the blood cholesterol increased between the ages of 3 and 5 months. Baker and Carrel (8), working with chicken serum, found that the average cholesterol content of that from 3 months old chickens was 225 mg. per cent while that from 4 to 5 years old chickens was 143 mg. per cent.

EXPERIMENTAL.

Cattle, guinea pigs, and rabbits were the species selected for the study and the results obtained will be given separately for each species. Bloor's method (9) was used for the total cholesterol determinations, and Bloor and Knudson's method (10) for combined cholesterol determinations. All animals used were on an adequate diet and all were bled within a period of 3 hours following feeding (new born calves and guinea pigs excepted).

TABLE II.

Calves That Had Suckled Dam at Time of First Bleeding.

Animal No.	Age.	Total serum cholesterol.	Combined serum cholesterol.	
		mg. per cent	*mg. per cent*	*per cent*
134, Holstein (male).	Between 5 and 6 hrs. old.	38	10	26
135, Holstein (male).	Between 5 and 6 hrs. old.	29	8	28
140, Holstein (female).	Approximately 6 hrs.	46	22	48
	48 hrs.	65	36	55
	6 days.	96	55	57
144, Jersey (female).	Approximately 6 hrs.	34	10	29
	48 hrs.	69	31	45
	6 days.	108	74	69

Cattle.

Six calves were used, blood being obtained from the external jugular vein. Two of the calves were available for only 1 day and two others for only 6 days after birth. The remaining two calves were available for a period of approximately 7 months. The results are given in Tables I and II.

Guinea Pigs.

Because of the small amount of blood that can be safely drawn from the heart of a single guinea pig, determinations were carried out on pooled samples of blood. From four to eight animals were

thus used for each determination. Animals included in the groups whose ages were less than 1 month had not yet been weaned, but all other animals were on the regular diet of hay, oats, and green clover. Only male animals were used. The results obtained are given in Table III.

Rabbits.

Blood was obtained from the marginal ear vein in the case of the rabbits used. A small litter was chosen for the work because

TABLE III.

Guinea Pigs.

Age.	Total serum cholesterol.	Serum cholesterol ester.		Average weight.
	mg. per cent	*mg. per cent*	*per cent*	*gm.*
1 hr.	64.5	Trace.		
5 days.	231.3	124.8	53.9	
20–30 days.	63.3	28.7	45.3	136
1–2 mos.	49.6	19.3	38.9	178
2–3 "	49.8	19.9	39.9	230
3–4 "	41.3	15.0	36.3	235
4–5 "	43.2	13.7	31.7	370
5–6 "	38.1	14.6	38.3	460
6–7 "	33.8	11.9	35.2	476
7–8 "	32.8	13.3	40.5	542
8–9 "	33.3	12.0	36.0	540
9–10 "	36.0	15.5	43.0	660
11–12 "	33.3	11.5	34.5	552
16–17 "	23.8	8.7	36.5	640
18–19 "	22.5	7.6	33.7	760
24–25 "	17.0	5.8	34.1	770
29–30 "	15.0	6.3	42.0	700

the animals reached a size large enough for bleeding at an earlier age. Two females and a male comprised the litter. Weaning was completed at 40 days of age. A separate litter of six animals was used in obtaining data regarding the serum cholesterol content of very young rabbits. These animals had nursed previous to the time the blood sample was obtained. They were so small that it was necessary to sacrifice them in order to obtain sufficient blood. The results are given in Table IV.

DISCUSSION.

The experimental data presented indicate that in herbivorous animals the changes in serum cholesterol as related to age are of two separate types. The first consists in a marked and rather rapid rise from birth for a relatively short period of time, varying with species, during the early life of the animal. The second con-

TABLE IV.

Rabbits.

Animal No.	Age.	Total serum cholesterol.	Serum cholesterol ester.		Weight.
	days	*mg. per cent*	*mg. per cent*	*per cent*	*gm.*
62 (male).	29	114.1	77.8	68.2	660
	45	96.3	53.4	55.4	1040
	65	54.0	21.3	39.4	1450
	134	42.3	5.8	13.7	2395
	162	34.0	8.3	24.4	2500
	292	20.0	9.3	46.5	2720
63 (female).	29	111.8	91.5	81.8	660
	45	104.8	63.4	60.5	1030
	65	62.6	21.7	34.6	1480
	134	76.6	38.3	50.0	3180
	162	58.6	25.6	43.7	2954
	292	52.6	28.0	53.2	3180
64 (female).	29	113.6	73.1	64.3	610
	45	95.2	41.6	43.7	1000
	65	65.0	19.0	29.2	1480
	134	75.6	19.8	26.2	3295
	162	66.0	19.1	28.9	2954
	292	55.6	26.6	47.8	3400
Pooled sample from litter of six rabbits.	3 hrs.	72.9	24.9	29.2	

sists in a less marked and more gradual decline with advancing age. The nature of the animal material available made it impossible to observe the period of increase more than casually in any but one species of animals, cattle, while the period of decrease was observed in guinea pigs, rabbits, and cattle.

The period of increasing serum cholesterol will be considered

first. Blood serum obtained from calves 1 hour after birth and before any colostrum had been taken contained only free cholesterol and that in relatively small amounts. Blood serum of calves between 5 and 6 hours old already contained more cholesterol than at birth and cholesterol was present in the combined as well as the free state. All animals bled at this time had had colostrum which in itself is quite rich in cholesterol and cholesterol ester, especially the first colostrum (11), and could very easily account for the almost immediate onset of the rise. The increase in both total and combined cholesterol was quite regularly progressive for a period of 3 weeks with the combined cholesterol increasing at a greater rate than the total cholesterol. From 3 weeks to around 10 weeks the serum cholesterol and cholesterol ester fluctuated irregularly but the ratio of combined cholesterol to total cholesterol continued to increase quite regularly. Following this and up to 200 days, the end of the experimental period, the serum cholesterol and cholesterol ester decreased quite regularly and progressively and the ratio of combined to total cholesterol decreased also. The small amount of data obtained on very young rabbits and guinea pigs indicates that these two species behave in a manner similar to cattle in that at birth the serum cholesterol content is low and rises subsequently to reach its highest level sometime under 1 month of age.

The period of declining serum cholesterol in guinea pigs and rabbits began some time under 1 month of age and was progressive and gradual. Guinea pig blood serum decreased in its cholesterol content from an average of 231 mg. per cent at 5 days of age to 15 mg. per cent in animals approximately 2½ years old. The cholesterol ester content of guinea pig serum decreased during the same period of time although in a less regular fashion. The per cent of total cholesterol existing in the combined form fluctuated irregularly and showed no constant changes.

The decline in serum cholesterol content in the case of rabbits was very regular for the only male animal included. Its serum cholesterol decreased from 114 mg. per cent at 29 days of age to 20 mg. per cent at 292 days of age. That the matter of sex is of some importance in determining variations in serum cholesterol with age is indicated by the results obtained with the two female rabbits. While their serum cholesterol decreased with age it did

so in a rather irregular fashion and failed to reach nearly as low a level as that of their male litter mate. The combined serum cholesterol decreased with age also and the percentage in the combined form tended to diminish up until the last bleeding when it rose again in all three animals.

The observation on rabbits regarding the effect of sex in determining variations in serum cholesterol with age is supported by preliminary unpublished experiments on guinea pigs. In these it was found that female animals showed such irregular fluctuations, unrelated in any way to age, that the use of only male animals was resorted to in later experiments.

Regarding cattle, the information at hand is limited to female animals. Both Calves 130 and 132 showed the period of decreasing serum cholesterol content observed in guinea pigs and rabbits and at the age of 199 days had reached the low levels of 93 and 91 mg. per cent respectively. That irregular fluctuations in serum cholesterol content, not related to age, would have been observed in these animals had it been possible to follow them further can be stated definitely, for, in determining the cholesterol content of large numbers of samples of blood serum from lactating cows never have values below 100 mg. per cent been observed. And in the great majority of instances they were much higher. Hence, at some time after 199 days of age, factors, other than age, would undoubtedly have entered to cause marked fluctuations in the serum cholesterol content. That these factors are rather intimately associated with reproduction and lactation is indicated by the observations of Shope and Gowen (12) on variations in the serum cholesterol of cattle during the "dry" period and during early lactation. From the differences observed to exist between the sexes as regards serum cholesterol variation with age it is strongly indicated that the cholesterol in blood serum serves more functions in female than in male animals.

The significance of the variations in serum cholesterol with age is not clear because of the general haziness of our ideas concerning the possible rôles of this substance in physiology. The data presented are insufficient to indicate whether the early increase in serum cholesterol is of exogenous or endogenous origin or whether the decrease in serum cholesterol observed to take place after the preliminary rise is, conversely, a matter of decreased

endogenous production or exogenous intake or both. Any discussion as to the mechanism or function of the changes observed would be little better than a surmise in the light of the deficiencies in our present knowledge concerning the physiological relationships of cholesterol.

SUMMARY.

1. Changes in serum cholesterol and cholesterol ester content as related to age are of two separate types. First, there is a marked and rather rapid increase from birth for a relatively short period of time during the early life of the animal. Secondly, there is a less marked and more gradual decline with advancing age.

2. At birth and before having received colostrum, sera from calves contain cholesterol only in the free form and that in very small quantities. Soon after nursing for the first time cholesterol ester appears in the blood serum and the total cholesterol content of the blood serum begins to increase.

3. Changes with age, in the serum cholesterol content, are more uniform and regular in male than in female animals.

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FEEDING EXPERIMENTS WITH A DIET LOW IN TYROSINE.

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INTRODUCTION.

Abderhalden (1) removed tyrosine from casein by crystallization, and fed the residue to an adult dog as the sole source of nitrogen. The animal rapidly lost weight. When this food was supplemented with tyrosine there was a gain in weight. Later Abderhalden (2) on feeding rats an amino acid mixture free from *l*-tyrosine found that the nitrogen balance immediately became negative. Upon addition of tyrosine or phenylalanine to the amino acid mixture the nitrogen balance rose, although equilibrium was not attained. This indicates that either tyrosine or phenylalanine is capable of supplying some carbocyclic compound which is required by the animal.

Totani (3) in similar experiments fed rats diets in which the nitrogen was supplied by hydrolyzed gelatin supplemented by amino acids. The addition of tyrosine to the food, believed to be otherwise complete, caused little or no increase in growth. Totani states that this must be explained in one or two ways,—either phenylalanine is capable of replacing tyrosine or animals have the power of synthesizing the benzene ring. Because of the small amount of phenylalanine in gelatin he believes the latter explanation to be the more probable.

Folin and Ciocalteu's (4) method makes possible more accurate information concerning the tyrosine content of experimental diets than has heretofore been available. Since hair hydrolysate is readily freed of a large portion of its tyrosine by crystallization, and because of its low phenylalanine content (5, 6) it was believed possible to obtain a diet adequate in other respects and yet very low in these two amino acids.

EXPERIMENTAL.

Twenty albino rats at 28 days of age were divided into two groups of ten rats each, in such a manner that litter mates were

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equally distributed between the two. The animals were fed for 12 weeks on diets that differed only in their tyrosine content. The nitrogen of these experimental diets was supplied by gelatin which had been digested by pancreatin, and acid-hydrolyzed human hair, supplemented by amino acids. The gelatin was prepared by dissolving 1 kilo in 5 liters of solution buffered at pH 7 by phosphates. 10 gm. of commercial pancreatin were added and the mixture kept at 38° for 4 days. The 4th day, more pancreatin was added and the digestion allowed to proceed for 4 more days, then placed in a refrigerator. After remaining in the refrigerator for 2 days, it was concentrated *in vacuo* to a thick paste, dried in a vacuum oven at 60°, and ground. Comparison of the values for total nitrogen (7) and amino nitrogen (8) indicates that about one-fourth of the total nitrogen in the gelatin existed as amino nitrogen at the end of the period of digestion. The hair hydrolysate was prepared by boiling the hair for 4 hours in concentrated hydrochloric acid. The mixture was concentrated *in vacuo* at 50° to a thick rubberlike mass. This was dissolved in water by warming on a water bath at a temperature not exceeding 60°. The solution was cooled in a freezing mixture, adjusted to a pH of 5.4, placed in a refrigerator for 48 hours, and then filtered rapidly by suction. The residue on the filter was washed with small quantities of ice water. All washings that did not give a positive reaction to Millon's reagent were added to the original filtrate. The filtrate was concentrated *in vacuo*, dried in a vacuum oven at 60°, and ground.

Diet I was prepared by mixing gelatin (8.8 per cent), hydrolyzed hair (4.5 per cent), tryptophane (0.2 per cent), histidine (0.2 per cent), cystine (0.1 per cent), tyrosine (0.2 per cent), dried yeast (3.0 per cent), salt mixture (4.0 per cent), butter (15.0 per cent), and dextrin (64.0 per cent). Diet II was prepared by omitting the tyrosine in the mixture and an increase of the digested gelatin content to 9.0 per cent. Due to the high chloride content of the hair hydrolysate the Osborne and Mendel (9) salt mixture was modified by omitting the sodium carbonate and hydrochloric acid. Analysis of the food mixture (10) showed a sodium content of 16 mg. per gm. Other data obtained by analysis of the food constituents, and calculations from these, expressing the concentration in the food mixture as fed, are to be found in Table I.

TABLE I.

Analyses of Food Constituents.

	Amino N (7).		Tyrosine (4).		Total N (4).	
	mg. per gm.	*mg. per gm. food mixture*	*mg. per gm.*	*mg. per gm. food mixture*	*mg. per gm.*	*mg. per gm. food mixture*
Hair hydrolysate	88.9	4.0	0.22	0.009	95.6	4.3
Hydrolyzed gelatin	43.3	3.8	0.48	0.042	162.4	14.3
Yeast			7.70			
Vegex			0.38			

TABLE II.

Summary of Animal Weights and Food Intake Record.

Rat No.	Initial weight.	Final weight.	Gain in weight.	Average food intake per wk.	Average food intake per 100 gm. rat.
			Diet I. Females.		
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
2	51.9	105.0	53.1	49.2	45.7
4	68.0	98.0	30.0	36.5	41.0
5	58.0	95.3	37.3	40.9	44.7
6	62.3	109.2	46.9	39.5	36.1
8	57.8	104.0	46.2	41.9	40.1
9	61.6	98.0	36.4	44.8	49.8
10	60.1	110.0	49.9	48.6	44.2
			Diet II. Females.		
15	56.1	101.0	44.9	42.8	40.2
16	58.6	83.5	24.9	31.2	37.2
17	51.1	96.6	45.5	41.9	44.4
			Diet I. Males.		
3	72.3	122.0	49.7	64.8	55.1
7	54.6	124.5	69.9	50.0	41.3
			Diet II. Males.		
13	65.4	188.3	122.9	72.3	40.0
14	67.5	195.0	127.5	56.8	30.3
19	62.5	128.5	66.0	59.8	48.7
20	62.0	103.5	41.5	48.3	48.7

Because the dried yeast did not appear to supply sufficient vitamin B, 1 mg. of Vegex was fed daily after the 3rd week. Animals 1, 11, 12, and 18 died within this period of vitamin B deficiency. Food intake records were made for the 3 final weeks of feeding. Averages of these weekly food intakes and the average

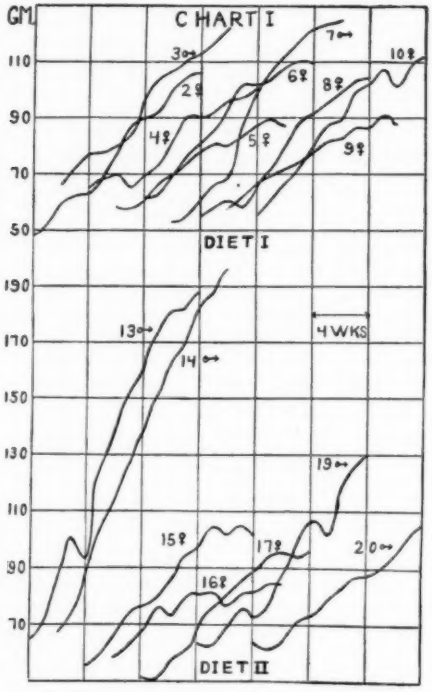

CHART I.

intake per 100 gm. rat are shown in Table II. Growth curves are shown in Chart I.

DISCUSSION.

The diets as prepared contained 39.7 mg. of tyrosine per 100 gm. of food. Of this 0.9 mg. was derived from the hair hydrolysate,

4.2 mg. from the gelatin, and 34.6 mg. from the yeast. The total nitrogen of the food is slightly in excess of 1.86 per cent. This is the equivalent of 11.55 per cent of protein. Calculated on the basis of average final weights the average daily intake of protein is 0.685 gm. per kilo of rat. The average food intake per week per 100 gm. rat is 44.29 gm. for those animals receiving Diet I, and 41.29 gm. for those receiving Diet II. The average food intake per animal per week is 46.2 gm. for those receiving Diet I and 50.4 gm. for those receiving Diet II. That the food intake is not the factor limiting growth is further shown by Rats 7, 13, and 14. Although these animals made the greatest growth, their food intakes were not the largest. The growth curves show marked variation within each dietary group, and the average final weight of the low tyrosine animals is 11.7 gm. greater than that of the Diet I animals. The variations within the groups, and the difference in final average weights is due to an unequal sex distribution. Thus two of the nine animals receiving Diet I were males, while five of the seven receiving Diet II were males.

SUMMARY.

Under the experimental conditions of the present study the growth of rats, for a period of 12 weeks, is independent of the tyrosine content of the food.

The authors wish to thank Dr. H. B. Lewis and Dr. R. C. Huston for their interest and advice during the course of this work.

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THE DURATION OF THE EFFECT OF ULTRA-VIOLET RADIATION ON CHICKENS.

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The present investigation was undertaken with the object of determining in a quantitative manner the duration of the effect of single doses of the ultra-violet radiation from an artificial source, the quartz mercury lamp. The percentage bone ash, and the calcium and inorganic phosphorus of the blood were selected as criteria to measure the duration of the effect. A search of the literature failed to reveal any report of a quantitative study of the effect of ultra-violet radiations on the chicken, although Hart (1) and his associates state that as little as 3 minutes per week of ultra-violet radiation from a quartz mercury lamp, through glass substitutes, brought about improved calcium assimilation in baby chicks.

Experimental Procedure.

360 14 day old White Leghorn chicks were distributed in six lots of 60 each, so that each reflected the individual weight distribution of the original lot. During the preexperimental period and throughout the experiment the ration supplied consisted of 99 per cent yellow corn plus 1 per cent sodium chloride, and liquid skimmed milk, the two foods being offered separately and *ad libitum* (2).

The ultra-violet radiations were from a Cooper-Hewitt poultry treater lamp, operated on 110 volts A.C., calculated D.C. voltage

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63 volts, arc wattage 270, placed so that the burner was 3 feet from the surface on which the birds stood. During an exposure they were kept in a circular cage, 3 feet in diameter, whose center coincided with that of the reflector of the lamp.

A sheet of the glass substitute, Cel-O-Glass, was interposed in the case of the lots which received a single exposure in order to prevent possible injurious effects of the shorter wave-lengths. This material does not transmit wave-lengths shorter than 2800 Å. The effectiveness of the radiation through this glass substitute is about one-third of that of the direct rays from a quartz mercury lamp (3). Thus an exposure of 45 minutes through it is equivalent to 15 minutes of the direct rays.

The six lots were given the following treatments: Lot 1, no radiation. Lots 2 to 5, single exposures, on the same day, of 45, 90, 180, and 270 minutes, respectively, through the glass substitute used as a filter. These are equivalent in the effect on bone formation to a direct exposure of approximately one-third of these periods. Lot 6, a daily exposure to 15 minutes direct radiation.

All of the lots were housed in a basement laboratory throughout the experiment. The only daylight which entered the room was from the northeast sky, through window glass.

Analytical Methods.

Selection for analysis was made by individual weights and the group selected represented the weight distribution of the lot under examination. The ash determinations were made of the individual, dried, alcohol-ether extracted femurs (os femoris) and large wing (humerus) bones (4). Calcium and phosphorus were determined in the serum by the Tisdall (5) modification of the Kramer and Tisdall method and the Briggs (6) modification of the Bell-Doisy method, respectively.

Discussion of Results.

Seven individuals were killed and analyses made to determine the condition of the whole group a few hours prior to the exposures. For the first 2½ weeks, the analyses of seven individuals from each lot, selected at half-weekly intervals, were used to determine the points on the curves; during the remainder of the

experiment five birds were selected at weekly intervals to determine each point. Analyses were made of individual bones and the results averaged. The blood was pooled for the calcium and phosphorus determinations.

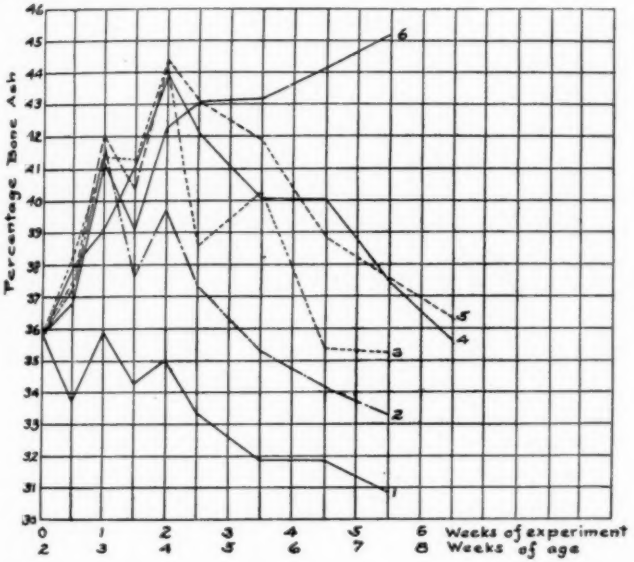

FIG. 1. Percentage of ash of thigh bones. Curve 1, no exposure to ultra-violet light. Curves 2 to 5 represent the effect of single exposures to filtered ultra-violet radiations of 45, 90, 180, and 270 minutes duration, respectively. Curve 6 shows the effect of 15 minutes of direct ultra-violet radiation daily.

That the ration was markedly deficient in vitamin D is amply demonstrated by Curves 1, Figs. 1 to 3, in the case of both the bone ash and blood calcium and phosphorus.

The sharp rise in the femur ash curves, Fig. 1, of the irradiated lots during the 1st week might be due to the fact that the ash percentage, 35.8 per cent, is lower than that observed at 2 weeks of age in an earlier experiment (4), in which the same technique was used, except that daily 15 minute radiations were begun at

5 days of age. In the earlier experiment the bone ash was 39.0 per cent at 2 weeks of age and 42.0 per cent at 3 weeks. Hence, in the present experiment, they reached what is probably normal at 3 weeks of age, at the end of the 1st week of experiment, having started from a lower bone ash content, due undoubtedly to a lack of the antirachitic factor during the first 2 weeks of life.

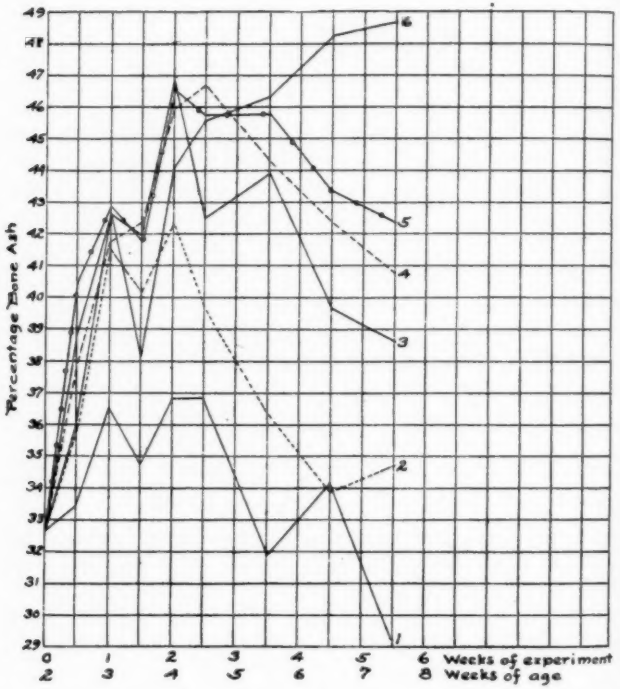

FIG. 2. Percentage of bone ash of wing bones (humerus). Curves 1 to 6, same as for Fig. 1.

The duration of the effect of the 45 minute exposure was for 1 week, after which the bone ash decreased as age, weight, and bone size increased. The rate of decrease is about the same as that of the basal lot. The bone ash of Lots 3 to 5 continued to increase for another week after which the decrease began. In the case of Lots 4 and 5, the decrease in each is at the same rate and

parallels roughly Lot 2 and the basal lot. Curve 3 is somewhat more irregular in its course than the others and its rate of decrease is a little more rapid than any of the other groups. According to the maxima reached, 90, 180, and 270 minutes of single exposure, each causes the same duration of the effect on the mineral

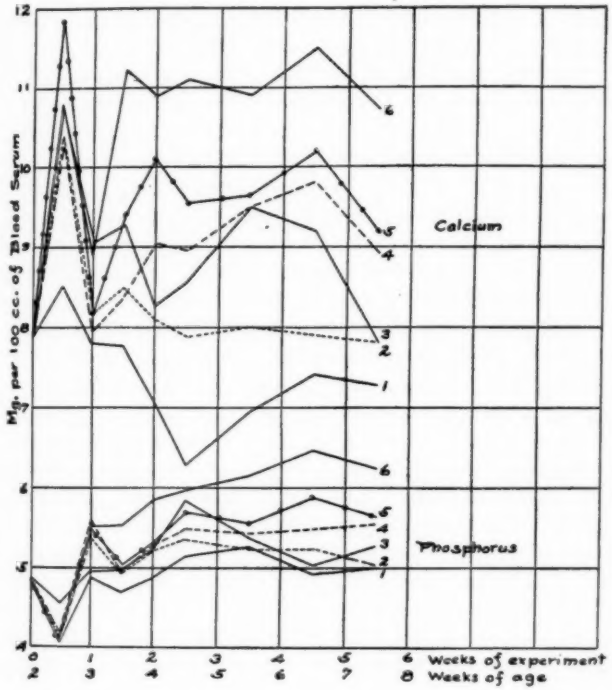

FIG. 3. Calcium and phosphorus of blood serum. Curves 1 to 6, same as for Fig. 1.

deposition in bones, but the rate of decrease of the curves suggests that the 180 and 270 minute exposures may have been slightly more effective than that of 90 minutes.

Results of the ash determinations of wing bones, Fig. 2, portray practically the same picture as do those of the femurs. As in

the case of the femurs, the percentage ash of the wing bones was lower at the beginning of the experiment than that of birds which had received an ample daily dosage of ultra-violet radiation. 40.5 per cent bone ash was observed at 2 weeks of age in an earlier experiment, when an ample daily radiation was given, while in this case it was 32.7 per cent at 2 weeks. At the end of the 1st week of the experiment all of the single exposure lots had reached a value of about 42.0 per cent, which is slightly lower than 43.5 per cent, obtained with ample radiation, and which is probably closer to the normal. At the close of the 2nd week, 4 weeks of age, the curves of the 90, 180, and 270 minute lots had reached a probably normal value for that age, 46 to 47 per cent, and the maximum for these lots. It is of interest that although the initial bone ash percentage of the wing bones is lower than that of the femurs, after the 1st half week, and in some cases at the 1st half week, the percentages are, almost without exception, higher throughout the experiment than those of the femurs. This has been observed in several earlier experiments.

On the same basal ration used in this experiment, but with 1.5 per cent of the corn replaced by Mead's cod liver oil, values of 10.5 mg. of calcium and 6.7 mg. of phosphorus per 100 cc. of serum were obtained at 2 weeks of age. Hence, as in the case of the percentage bone ash of the femurs and wing bones, the initial values shown in Fig. 3 are lower than normal, undoubtedly because of a deficiency of the antirachitic factor during the first 2 weeks of life. The response to the 270 minute exposure is more outstanding in the case of the calcium values than that of those of shorter duration and than the effect on the bone ash. The calcium level of the blood, on the ration used, is probably a more flexible quantity. Although the blood picture, Fig. 3, does not portray the duration of the effect of a single exposure as do the bone ash values, the levels assumed by the curves are, in most instances, of the order of the duration of the exposures.

The sharp increase in the calcium value of each of the radiated lots was followed by a marked drop after which there was another rise to a fairly constant level for several weeks, the curve of Lot 3 showing the greatest fluctuation. Likewise, the curves of the phosphorus values tended toward a constant level as the experiment progressed.

During the first 2 weeks of the experiment, when the fluctuations of the curves were most marked, those of the calcium values were in general opposed to those of phosphorus and the bone ash. The data do not permit a definite interpretation of the relation of these fluctuations to each other, but the sharp rise in the calcium curves and the coincidental drop in those of the phosphorus take place during the time that the mineral content of the bones is increasing rapidly, suggesting an association with the process of bone formation.

Lot C, which received 15 minutes per day of the direct radiation, was used in order to compare the effects of the various single exposures with the usual procedure of daily irradiation. The response during the 1st week of the experiment was the same as that of the single exposure lots, but the bone ash values are slightly less than those of the 90, 180, and 270 minute lots until the end of 2½ weeks of experiment, after which the bone ash of the daily exposure lot continues to increase. During the first 3½ weeks of the experiment the fluctuations of the daily exposure lot, and during the first 2½ weeks those of the basal lot, parallel those of the single exposure groups. Hence, environmental factors common to all six lots may be in part the cause of these fluctuations.

The observations made in this study suggest the possibility of the use of single exposures at intervals of several days, rather than daily exposures in the practical applications of ultra-violet radiations.

SUMMARY.

1. The bone ash percentages demonstrate that the duration of the effect on bone formation of a single exposure of 45 minutes to the filtered radiation (equivalent to 15 minutes of the direct) from a quartz mercury lamp at a distance of 3 feet is 1 week and that for single exposures of 90, 180, and 270 minutes it is 2 weeks.

2. The amount of calcium and inorganic phosphorus of the blood varies directly with the length of the exposure to ultra-violet radiations.

3. The values of the calcium and inorganic phosphorus of the blood fluctuate with relation to the curves of the percentage

bone ash in a manner that suggests a relationship to the process of mineral deposition in the bones.

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INACTIVATION OF INVERTASE AND RAFFINASE BY HEAT.*

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The present authors have had occasion partially to inactivate some preparations of yeast invertase by heat. Upon comparison of the activity of the preparations in the hydrolysis of sucrose and the sucrose part of raffinose, before and after the partial inactivation, it was found, in the case of some of the preparations, that there was a greater loss in activity when the latter was calculated from rate of hydrolysis of raffinose than when calculated from the rate of hydrolysis of sucrose. For example the preparation, by means of which the data given in Table I were obtained, suffered a loss of 58 per cent in activity with respect to the hydrolysis of sucrose, while based on the change in activity towards raffinose, the loss amounted to 75 per cent. Several other preparations of yeast invertase showed similar behavior, although in some cases the decrease in activity was the same with respect to both sugars. Why the loss in activity of the different enzyme preparations due to heating was different with respect to the two sugars in some cases and not in others, the present results do not show.

Experimental Procedure.

The procedure followed in obtaining the results given in Table I was as follows: A top yeast (Race-Gebrüder Mayer, *Saccharomyces cerevisiæ*) was grown in molasses, filtered, and to the solid mass of yeast thus obtained, toluene was added, and then the mixture was allowed to stand in a glass bottle and autolyze for about a year at room temperature. The autolyzed material was filtered and the filtrate dialyzed in collodion bags against running

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tap water for 10 days. The dialysate thus obtained was clarified by filtration through infusorial earth. To about 200 cc. of the clear enzyme solution was added sufficient 0.02 M sodium citrate

TABLE I.

Experiment I. 150 cc. 5 per cent sucrose solution, containing 25 cc. unheated enzyme Solution A and 0.02 M sodium citrate buffer. pH 5.0. Samples 25 cc. Temperature of hydrolysis and polariscope readings 25°.

Time, *min.*	31	75	132	182	30	90	153	190
Change in rotation.	0.11°	0.24°	0.42°	0.59°	0.10°	0.31°	0.52°	0.62°

Initial velocity. 0.003306°; corrected velocity, 0.011009° per min.

Experiment II. 150 cc. 5 per cent sucrose solution, containing 25 cc. heated enzyme Solution B. Other conditions as in Experiment I.

Time, *min.*	36	111	189	229	44	113	171	190
Change in rotation.	0.15°	0.49°	0.84°	1.03°	0.20°	0.49°	0.81°	0.85°

Initial velocity. 0.004622° per min.

Loss in activity. (From Experiments I and II) 58 per cent.

Experiment III. 150 cc. 2.05 per cent hydrated raffinose solution, containing 50 cc. unheated enzyme Solution A. Other conditions as in Experiment I.

Time, *min.*	80	135	196	271	70	124	154	181
Change in rotation.	0.125°	0.21°	0.325°	0.375°	0.105°	0.18°	0.23°	0.28°

Initial velocity. 0.001525°; corrected velocity 0.005078° per min.

Experiment IV. 150 cc. 2.05 per cent hydrated raffinose solution, containing 50 cc. heated enzyme Solution B. Other conditions as in Experiment I.

Time, *min.*	93	168	230	282	58	107	167	243
Change in rotation.	0.11°	0.20°	0.29°	0.33°	0.08°	0.14°	0.21°	0.30°

Initial velocity. 0.001250° per min.

Loss in activity. (From Experiments III and IV) 75.4 per cent.

The initial velocity in Experiments I and III was multiplied by 3.33 in order to correct for the more dilute concentration of the enzyme in the unheated Solution A as compared to Solution B.

solution to give an acidity of pH 5.6. The enzyme solution was then divided into two equal portions, and one of these was placed in a thermostat and heated at 60° for an hour.

Enzyme Solution A.—10 cc. of the unheated portion were di-

luted to 1 liter by the addition of 0.02 M sodium citrate solution. This enzyme solution possessed a pH value of 5.0.

Enzyme Solution B.—30 cc. of the above heated portion were diluted to 900 cc. with the same 0.02 M sodium citrate solution as was used for Solution A (pH 5.0). Solution B was made more concentrated than Solution A in order to make the slower rates of hydrolysis more convenient to read. Since the rate of hydrolysis is proportional to the concentration of the enzyme, this difference in dilution was taken into account in the corrected velocity values given in Table I.

Determination of Loss in Activity by Comparing Rates of Hydrolysis of Sucrose by Heated and Unheated Enzyme.—25 cc. portions of the enzyme Solutions A and B were added respectively to each of two 125 cc. sucrose solutions. The sucrose solutions had been prepared previously by dissolving 7.5 gm. of sucrose in sufficient 0.02 M sodium citrate solution to give a final volume of 125 cc. (same citrate as used in making up Solutions A and B). All the solutions, both enzyme and sugar, were kept in the thermostat at 25°, the temperature at which the hydrolyses were run, for some time before the enzyme was added to the sugar solutions.

The concentration of the enzyme in the sugar solutions undergoing hydrolysis was so small that the rate of hydrolysis was very slow, thereby permitting the taking of a sample for the initial reading a minute or two after the reaction had started. 25 cc. samples were taken from time to time and the hydrolysis stopped in each sample by the addition of a drop of saturated sodium hydroxide solution. The readings were made in 200 mm. polariscope tubes at 25° temperature, with a mercury quartz lamp as the source of light.

The velocities of hydrolysis given in Table I are given in degrees change in rotation per minute. These values were obtained as follows: The polariscopic reading of each sample taken from the solution undergoing hydrolysis was subtracted from the reading of the first sample taken at the beginning of the hydrolysis. The change in rotation thus obtained was plotted as ordinates against the corresponding time of hydrolysis. By drawing a tangent to this curve at its zero point, the value of the initial velocity, the velocity at the beginning of the hydrolysis, when very little invert sugar was present to influence the reaction, was obtained in

degrees change in rotation over minutes, or degrees change per minute.

Determination of Loss in Activity by Comparing Rates of Hydrolysis of Raffinose by Heated and Unheated Enzyme.—50 cc. portions of a raffinose solution were added to 100 cc. portions of each of the two enzyme Solutions A and B. The raffinose solution was prepared by dissolving 6.766 gm. of hydrated raffinose in 110 cc. of 0.02 M sodium citrate solution. The rest of the procedure was the same as that followed in the case of the sucrose hydrolyses.

The hydrated raffinose used gave $[\alpha]_{546}^{25} = +122.4°$. The enzyme solution contained no melibiase. 100 cc. of a solution containing 2 gm. of hydrated raffinose, 25 cc. of the original undiluted enzyme solution (the same solution as used in preparing Solution A), and 0.02 M sodium citrate buffer, were permitted to stand for several days until constant rotation was attained. The rotation of the raffinose solution before hydrolysis was +4.94° and this dropped to +2.52°. Calculated change in rotation, if the raffinose had been hydrolyzed to melibiose and fructose, is 2.54°, and if to galactose, glucose, and fructose then it is +0.48°. (Mercury light was used.)

The fact that there is a difference in the degree of inactivation of the invertase and raffinose when a yeast enzyme solution is heated, may have some bearing upon the question whether or not these two enzymes in yeast are identical. Several papers have appeared recently dealing with this question, and the claim is now made by Willstätter and Kuhn (1) and by Josephson (2) that the two enzymes are the same. The question is also discussed in some of the more recent books dealing with enzyme chemistry (3).

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EXPERIMENTS ON THE NUTRITIVE PROPERTIES OF GELATIN.

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Numerous attempts have been made in the past to supplement gelatin with amino acids in such a fashion as to render it capable of maintaining body weight or of preserving nitrogen equilibrium. Most of these efforts have met with complete failure. A few, notably those of Kauffmann (1905) and Abderhalden (1912), appear at first glance to have been somewhat more successful. An examination of the data of these authors, however, reveals the fact that the periods upon gelatin diets during which nitrogen equilibrium was approximated were of exceedingly short duration. Even if it be granted that gelatin with suitable amino acid additions suffices for the maintenance of nitrogen equilibrium in animals for periods of 5 to 10 days, one would scarcely be justified in assuming on the basis of such evidence that the same would be true for longer periods of time. Furthermore, most of the studies of the nutritive properties of gelatin were made before the recognition of the importance of the vitamins as dietary factors.

Since the development of the newer aspects of nutrition during the past two decades very few experiments upon gelatin have been reported. In their classical studies with isolated proteins, Osborne and Mendel (1912–13) showed that this substance is quite incapable of serving as the sole source of nitrogen. Rats placed upon diets of gelatin, starch, lard, and "protein-free milk" rapidly declined in weight, but when half of the gelatin was replaced by casein or gliadin, recovery with maintenance or growth ensued. Osborne and Mendel did not attempt to correct the deficiencies of gelatin by the addition of individual amino acids, but they point out (p. 242) that the "failure to be maintained . . . . is not to be ascribed to a failure in utilization; for nitrogen determinations made on the feces showed that the utilization of the protein was good."

* Most of the experimental data in this paper are taken from a thesis submitted by Richard W. Jackson in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

Later Totani (1916) attempted to render gelatin adequate as a source of nitrogen for rats by supplementing the diets with purified amino acids. He reports that the addition of tryptophane alone, or of tryptophane with tyrosine, is without influence upon the quality of the nitrogen. Animals upon gelatin diets, either with or without the supplementing amino acids, suffer 40 per cent losses in body weight in the course of a single month. The author states that hydrolyzed gelatin proved to be a much better source of nitrogen inasmuch as rats receiving this material experienced losses of only 18 per cent in a month. Furthermore, the inclusion of tryptophane in the hydrolyzed gelatin diet led to approximate maintenance. The further addition of tyrosine did not lead to better growth than was secured with tryptophane alone. When tryptophane, tyrosine, cystine, and histidine were incorporated in the ration the results are said to have been "somewhat more satisfactory." The author concludes (p. 397) that, "In the case of the rat, the nutritive efficiency of gelatin is greatly increased by previous hydrolysis. Gelatin when fed intact appears to be badly digested and absorbed." This statement is not in accord with the experience of Osborne and Mendel as outlined above. It is also contrary to the findings of numerous investigators who have studied the utilization of gelatin in animals other than the rat. Thus Reilly, Nolan, and Lusk (1898) report that in diabetic dogs sugar is formed from gelatin in about the same amount as from other proteins. McCollum (1911–12) and Robison (1922), in experiments upon the pig and man respectively, have shown that the ingestion of relatively large quantities of gelatin increases the fecal nitrogen very slightly over the starvation output. Even in the rat, McCollum, Simmonds, and Pitz (1917) have demonstrated that gelatin is capable of supplementing the proteins of rolled oats in a remarkable fashion. These and other experiments reported in the literature would seem to indicate quite conclusively that whatever may be the nutritive deficiencies of gelatin they are not associated with an incapacity of the alimentary enzymes to accomplish its digestion.

In connection with certain investigations in this laboratory we have undertaken a further study of the inadequacies of gelatin as a source of nitrogen for maintenance and growth. On the basis of the most reliable analyses of gelatin it would seem that the amino acid deficiencies are quite evident and could be easily remedied. That the problem is not so simple as it appears will be brought out in the following pages.

Early in our work we observed that the addition of the three conspicuously absent amino acids, cystine, tryptophane, and tyrosine, did not give uniformly the growth desired. It was decided, therefore, to extend the scope of the investigation so as to include not only the amino acid deficiencies, but any other factors

which conceivably might alter the nutritive value of the protein. The following are some of the questions which suggested themselves:

1. Since the very careful and complete analysis of gelatin made by Dakin (1920) reveals the absence of valine, hydroxyglutamic acid, and isoleucine, it seemed not improbable that one or more of these might be limiting factors.

2. Inasmuch as certain other components of gelatin are present in smaller amounts than occur in casein, it appeared necessary to determine the influence upon growth of suitably increasing their intake, either by adding the pure amino acids themselves, or by raising the level of the gelatin in the diet.

3. The pronounced disproportion in the distribution of amino acids in gelatin as compared with casein, particularly as regards the high content of glycine and alanine in the former, suggested that an *improper balance* between the amino acids might exert an influence upon nutrition. Evidence for or against this conception might be secured by incorporating in an otherwise satisfactory diet amounts of glycine and alanine comparable to those present in gelatin.

4. It has been the experience of many that the ingestion by the human subject of large quantities of gelatin is exceedingly difficult. Indeed, depression and nausea almost invariably result. In commenting upon this observation Howell (1925, p. 288) says, "A fact of this kind indicates that the sensory apparatus of the appetite is influenced in some specific way by the metabolism of this particular material." In order to determine whether the peculiar physical or chemical nature of gelatin exerts a deleterious effect upon the organism, we have followed the growth of animals which received a third of their diet in the form of this protein in addition to an abundance of nitrogen of good quality.

5. If gelatin contains amino acid linkages incapable of cleavage by the digestive enzymes, complete hydrolysis of the material before its incorporation in the diet should improve the quality of the nitrogen, as is reported to be the case by Totani (1916). A reinvestigation of this point seemed necessary.

EXPERIMENTAL.

White rats served throughout as the experimental animals. Invariably they were placed upon a casein diet for brief periods at the beginning of the experiments in order to test their growth propensities. In view of experience gained in the course of the

TABLE I.

Composition of Diets.

Diet	A.	B.	C.	D and E.*	F.	G.	H and I.*
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Casein	18.0	18.0	18.0				
Gelatin			35.0	35.00	30.00	25.00	15.00
Dextrin	34.0	22.0		15.30	16.30	20.90	24.00
Sucrose	15.0	15.0	14.0	15.00	15.00	15.00	15.00
Salt mixture	4.0	4.0	4.0	4.00	4.00	4.00	4.00
Agar	2.0	2.0	2.0	2.00	2.00	2.00	2.00
Cod liver oil	5.0	5.0	5.0	5.00	5.00	5.00	5.00
Lard	22.0	22.0	22.0	22.00	22.00	22.00	22.00
Tyrosine (*l*)				0.90	0.90	0.90	0.90
Cystine (*l*)				0.50	0.50	0.50	0.50
Tryptophane (*l*)				0.35	0.35	0.35	0.30
Histidine monochloride (*l*)					0.26	0.32	0.40
Valine (*dl*)					2.70	2.70	4.00
Phenylalanine (*dl*)					0.60	0.80	2.00
Glutamic acid (*d*)					2.10	2.40	4.00
Aspartic " (*l*)							1.00
Leucine (*dl*)							1.33
Isoleucine (*dl*)							1.33
Norleucine (*dl*)							1.33
Glycine		9.0					
Alanine (*dl*)		6.0					

* Diets E and I contained hydrolyzed gelatin but in other respects were exactly like Diets D and H respectively. With the exception of Diets E and I, all of the gelatin diets contained the protein in an unhydrolyzed form.

investigation to the effect that younger animals do not seem to withstand the change to the gelatin diets as satisfactorily as do older ones, we permitted all rats employed in the experiments reported herein to attain weights of at least 100 gm. before being transferred from the casein ration. In the conduct of the experiments and in the care of the animals essentially the methods of

Osborne and Mendel as described by Ferry (1919–20) were followed.

The composition of the diets is shown in Table I. In addition to the articles of food enumerated, vitamin B was furnished to each animal daily in the form of tablets containing 50 mg. of commercial yeast extract or 200 mg. of whole yeast.[1] The gelatin was a special product[2] which in manufacture had not been subjected to the action of bleaching agents. It consisted of white, opaque flakes, which were thoroughly dried *in vacuo*, ground, and passed through a 40 mesh sieve. A 1 per cent solution exhibited a faint Millon test, and a negative Hopkins-Cole reaction. Hydroferrocyanic acid gave a very faint turbidity.

The hydrolyzed gelatin employed in Diets E and I was prepared exactly according to the procedure followed by Dakin (1920) in the analysis of this protein with a recovery of over 90 per cent of the component amino acids. For this purpose, a mixture composed of 250 gm. of gelatin, 300 gm. of concentrated sulfuric acid, and 650 gm. of water was heated to boiling for 20 hours at atmospheric pressure. The resulting solution was autoclaved at 135–140° for 8 hours. After dilution, the sulfuric acid was removed with barium hydroxide, and the filtrate and washings from the barium sulfate were combined and evaporated to dryness. The final material, after grinding, was light brown in color.

The inorganic portion of the rations was supplied in the form of Osborne and Mendel's (1919) salt mixture. Of the amino acids used in supplementing the diets, tyrosine, cystine, tryptophane, histidine monochloride, glutamic acid, and glycine were prepared in this laboratory from proteins. The aspartic acid was the natural levo variety secured from Kahlbaum. The valine, phenylalanine, leucine, isoleucine, norleucine, and alanine were racemic compounds most of which were synthesized in the organic laboratory of this University. Both the natural and racemic acids were recrystallized until they gave theoretical values for total or

[1] The extract was prepared by the Harris Laboratories, Tuckahoe, N. Y., while the whole yeast was secured from the Northwestern Yeast Company, Chicago. Both the extract and the yeast were thoroughly tested as to their growth-promoting properties before being employed in the gelatin experiments.

[2] Secured from the Wilson Laboratories, Chicago.

amino nitrogen. Whenever a racemic acid was used, twice the calculated amount was incorporated in the ration so as to insure the presence of the active enantiomorph in the quantity desired.

In supplementing the gelatin diets we have used the composition of casein as our guide. On the basis of the most reliable figures for casein composition (*cf.* Mendel, 1923, p. 115), and the analysis of gelatin by Dakin (1920), we have calculated the quantity of each known amino acid[3] present in 100 gm. of food containing 18 per cent of casein and 35 per cent of gelatin respectively. These figures are presented in the first two columns of Table II. In the other columns of this table are shown similar data for the supplemented gelatin diets employed in this investigation.

In most of our experiments gelatin was incorporated in the food at a 35 per cent level. This relatively high proportion was adopted instead of the customary 18 per cent because the protein in question contains several amino acids in quantities far in excess of the accepted values for casein. A comparison of the composition of the two proteins indicates that gelatin yields superfluous amounts of glycine, alanine, hydroxyproline, and arginine. Even if we assume that quantities of these amino acids considerably larger than are supposed to be present in casein are required by the organism, we are still confronted with the necessity of feeding almost double the proportion of gelatin as compared with casein in order to insure the presence of the other amino acids in approximately comparable amounts. A diet containing 35 per cent of gelatin seemed, therefore, a reasonable starting point.

Growth on a Gelatin Diet Supplemented with Three Amino Acids.—The data presented in Table II make clear that in addition to tyrosine, cystine, and tryptophane, gelatin is wanting also in valine, isoleucine, and hydroxyglutamic acid. Furthermore, on a 35 per cent basis, phenylalanine, glutamic acid, and histidine

[3] During the progress of this investigation methioneine, the new sulfur-containing amino acid isolated from casein by Mueller (1923), was identified by Barger and Coyne (1928). For obvious reasons this substance was not taken into consideration in our experiments. Mueller reports its presence in gelatin, but apparently in much smaller quantities than in other proteins. At a later date we expect to determine the relation of methioneine to the nutritive properties of gelatin.

are somewhat below the levels present in the 18 per cent casein diet (Diet A) taken as our standard. In our first experiments, we have supplied only tyrosine, cystine, and tryptophane. Thus the resulting gelatin diet (Diet D) was presumably devoid of valine, isoleucine, and hydroxyglutamic acid. There existed also the possibility that it might be somewhat inadequate in respect

TABLE II.

Amino Acids Present in 100 Gm. of the Various Diets as Compared with 35 Per Cent of Unsupplemented Gelatin.*

	35 per cent gelatin.	Diet A.	Diets D and E.	Diet F.	Diet G.	Diets H and I.
	gm.	gm.	gm.	gm.	gm.	gm.
Glycine	8.93	0.08	8.93	7.65	6.38	3.83
Alanine	3.05	0.33	3.05	2.61	2.18	1.31
Valine	0	1.43	0	1.35	1.35	2.00
Leucine	2.49	1.75	2.49	2.13	1.78	1.73
Isoleucine	0		0	0	0	0.66
Norleucine						0.66
Serine	0.14	0.09	0.14	0.12	0.10	0.06
Phenylalanine	0.49	0.70	0.49	0.72	0.75	1.21
Tyrosine	?	0.81	0.90	0.90	0.90	0.90
Proline	3.33	1.37	3.33	2.85	2.38	1.43
Hydroxyproline	4.94	0.04	4.94	4.23	3.52	2.12
Aspartic acid	1.19	0.74	1.19	1.02	0.85	1.51
Glutamic "	2.03	3.92	2.03	3.84	3.85	4.87
Hydroxyglutamic acid	0	1.89	0	0	0	0
Histidine	0.32	0.45	0.32	0.47	0.49	0.46
Arginine	2.87	0.69	2.87	2.46	2.05	1.23
Lysine	2.07	1.37	2.07	1.77	1.48	0.89
Tryptophane	0	0.27	0.35	0.35	0.35	0.30
Cystine	?	Present.	0.50	0.50	0.50	0.50

* The figures are for the *active* amino acids. When racemic acids were employed, only half of each quantity added to a diet is included in the above data.

to phenylalanine, glutamic acid, and histidine. The absence of hydroxyglutamic acid was disregarded in all of our experiments inasmuch as Osborne, Leavenworth, and Nolan (1924) have pointed out that edestin, which does not contain this amino acid, is capable of supporting normal growth.

Of twenty-six rats placed upon Diet D, thirteen died at intervals

varying from 9 to 48 days. The curves representing the body weight changes of two of the animals which failed to survive (Rats 138 and 150) are reproduced in Chart I, and are typical of all except for the length of time which elapsed before death. The curves show profound nutritive failure accompanied by

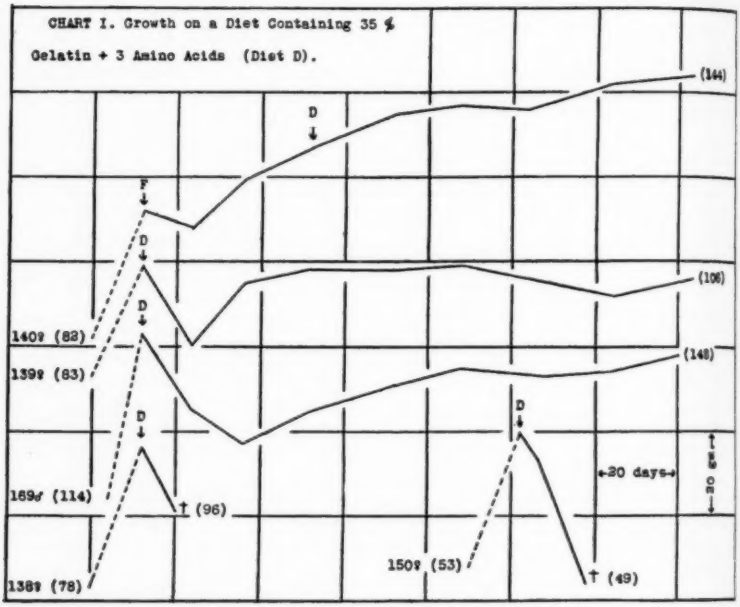

CHART I. The numbers in parentheses signify the initial and final weights of the rats. The broken line portion of each curve represents the growth of the rat during the preliminary period upon Diet A. The solid lines show the growth upon the experimental rations. The arrows denote the points at which the dietary changes were made. The diets are designated by the letters above the arrows.

amazingly rapid losses in weight. In some instances the food consumption dropped almost to zero during the last few days preceding death. At autopsy most of the animals which had died displayed badly deteriorated kidneys. Hemorrhagic lesions were observed in one case, while the exteriors of the kidneys of several

others showed soft, pale, and pitted areas. We are inclined to believe that the renal involvement, rather than the nutritive failure *per se*, was responsible for the early fatal outcome in so many of our experiments. Nor does it seem likely that this can be attributed primarily to the presence of the free amino acids in the ration since numerous experiments in this laboratory (Rose and Huddlestun, 1927) have shown that the incorporation in casein diets of 1.5 per cent of tyrosine, cystine, or tryptophane does not inhibit growth or induce renal failure in rats of 100 gm. weight.[4] Apparently, the effects outlined above are associated with the gelatin factor.

The thirteen animals which survived the transfer to the gelatin ration showed variable but usually very rapid losses in weight immediately after the dietary change, but subsequently improved. We believe that they also experienced kidney injury, but happened to be better adapted to or more successful in adjusting themselves to the adverse conditions than were their fellows who succumbed. The growth curves secured in three long continued experiments are reproduced in Chart I. The typically rapid decreases in weight on the gelatin ration are seen in the curves of Rats 139 and 169. These animals lost 19 and 26 gm. respectively before recovering from the effects of the ration. Subsequently, slow growth followed by practical maintenance ensued. The experiment upon Rat 140 was slightly different from those upon Rats 139 and 169 in that the former animal was transferred from the normal diet to one containing 30 per cent of gelatin supplemented with seven amino acids (Diet F). After it had remained upon this ration for 40 days, Diet D was substituted. This rat showed a smaller decrease followed by a greater increase in weight than either of the others. When the experiment was discontinued at the expiration of 132 days, a net gain of 32 gm. had been made since the transfer from the casein ration.

In Chart II are reproduced growth curves secured in shorter experiments with Diet D. The rats were litter mates. The data are of interest as illustrations of the wide individual differences

[4] Recent unpublished experiments by G. J. Cox of this laboratory have shown that rats weighing 40 to 50 gm. each are rendered nephritic by cystine administration. Under identical conditions, animals of 100 gm. weight manifest no ill effects.

which one encounters in dietary studies involving the use of gelatin. Four of the animals of this litter (curves not shown) died within 2 weeks after the change to Diet D. Two (Rats

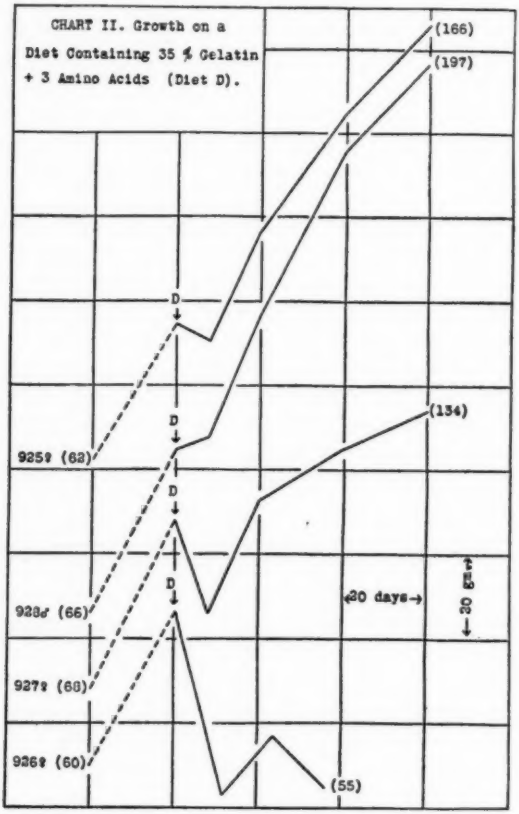

CHART II.

926 and 927, Chart II) underwent profound losses, but Rat 927 partially recovered later. Rats 925 and 928 experienced only slight decreases in body weight followed by almost normal growth. The latter animals, though constituting a small percentage of the

total number employed, are of particular significance in that they prove that very good growth *is possible* when gelatin supplemented with tyrosine, cystine, and tryptophane serves as the sole source of nitrogen except for the small quantity present in 200 mg. of yeast. On the other hand, it is quite evident that Diet D permitted rapid increases in weight in *only two out of a total of twenty-six animals.* This fact led us to try the influence of other proportions of gelatin, and of other supplementing amino acids.

Growth on Gelatin Diets Supplemented with Seven and Eleven Amino Acids.—Inasmuch as tyrosine, cystine, and tryptophane, as stated above, are not the only factors which are low in gelatin diets, attempts were made to improve the quality of the protein by the further addition of histidine, valine, phenylalanine, and glutamic acid. For this purpose, two diets were formulated (Diets F and G, Table I) containing 30 and 25 per cent of gelatin respectively. The data in Table II show that presumably each contained adequate amounts of all of the amino acids definitely known to be present in casein with the exception of isoleucine and hydroxyglutamic acid. Since the latter may be disregarded for reasons already stated, the absence of isoleucine is the only possible deficiency in the rations in so far as our knowledge of the composition of gelatin and casein permits one to judge.

Four rats were placed upon each of these diets after preliminary periods upon Diet A. To our surprise the growth curves were not better than those obtained with Diet D. Of the eight animals two died within 16 days. The experiments upon three others were discontinued after the animals had lost weight very rapidly, and were in a greatly weakened condition. Three recovered from the injurious effects of the gelatin and made slow gains. One of these (Rat 140) was subsequently changed to Diet D and has already been referred to in connection with Chart I. The graph makes evident the fact that the growth of this animal was not altered significantly by the transfer from Diet F containing seven supplementing amino acids to Diet D containing only three. The growth curves of the other animals upon Diets F and G (with the exception of Rat 141) are omitted from the charts as they add nothing to the solution of the problem in question.

Rat 141 was changed from Diet F to Diet H containing 15 per cent of gelatin supplemented with eleven amino acids. An

inspection of Table I reveals the fact that in addition to the seven amino acids included in Diet F, Diet H contained the three leucines and aspartic acid. The growth curve is shown in Chart III. The animal made a slow but fairly consistent gain upon this ration, and when the experiment was discontinued had increased in body weight to the extent of 24 gm. in 72 days. At a later

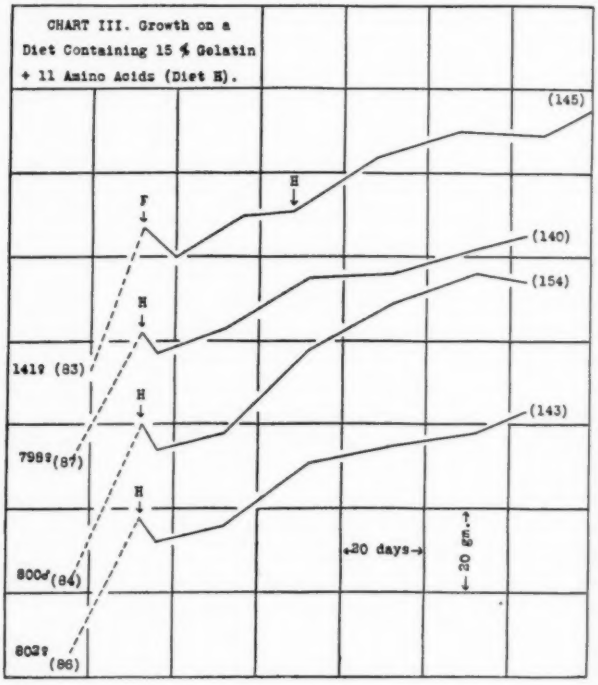

CHART III.

date, three other rats (Rats 798, 800, and 802) of a single litter were transferred directly from the casein ration to Diet H. The growth curves of these animals also are shown in Chart III, and manifest increases in body weight comparable to that attained by Rat 141. The only striking difference between these experiments and those involving the use of the 35 per cent gelatin diet is the fact

that upon the lower level of gelatin none of the animals succumbed. After becoming "adjusted" to Diet H, all of the rats made moderate gains. It should be emphasized that Diet H contained all known amino acids with the exception of hydroxyglutamic acid. Aside from the latter, the only component present in distinctly lower quantity than in an 18 per cent casein diet is lysine. That this was not a limiting factor is made probable by the observation of Osborne and Mendel (1915) that edestin, which contains 1.65 per cent of lysine, supports normal growth when included in the ration at a 15 per cent level. Under such circumstances, 100 gm. of the edestin diet carry only 0.25 gm. of lysine, or less than one-third the amount present in our Diet H.

Attempts to improve the quality of gelatin rations by increasing the proportion of the protein gave even less satisfactory results than were secured with 15 to 35 per cent levels. Of three rats placed upon a diet containing 55 per cent of gelatin supplemented with tyrosine, cystine, and tryptophane, two died promptly after very rapid losses in weight, and the third was moribund when the experiment was discontinued. Further studies of this sort seemed very unpromising and were abandoned.

Does Gelatin Exert a Deleterious Action?—In order to determine whether gelatin, or the unusually large quantities of glycine and alanine which it contains, is detrimental to the organism two modifications of Diet A were formulated, one containing 9 per cent of glycine and 6 per cent of racemic alanine (Diet B), and the other containing 35 per cent of gelatin (Diet C). The added components replaced equivalent amounts of carbohydrate without altering the ratio of casein. Thus each ration carried an adequate quantity of protein of suitable quality (casein) in addition to the superfluous nitrogenous materials. After preliminary periods upon Diet A, two rats were placed upon Diet B, and three upon Diet C. The results are shown in Chart IV. All five animals, but especially those upon Diet C, exhibit decreases in growth rates after the transfer from the normal ration. Rat 188, one of those which received the diet containing 35 per cent of gelatin, experienced a loss in weight of 17 gm. in 8 days before becoming adjusted to the new dietary régime. Following recovery, rather rapid growth ensued in all cases. The experiments indicate that neither gelatin nor a mixture of two of its component

amino acids permanently prevents growth when incorporated in an otherwise normal diet. On the other hand, the large proportion of gelatin (35 per cent) may produce a *temporary inhibition*

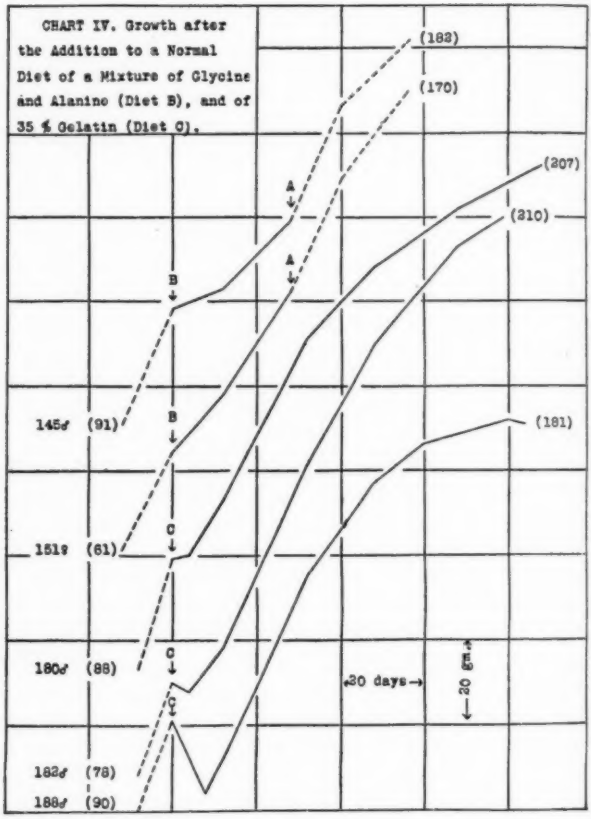

CHART IV.

in growth despite the presence of a suitable amount of adequate protein. This fact, when considered in conjunction with the observation that renal injury frequently followed the administration of the high gelatin diet (Diet D), renders it difficult to

escape the impression that this protein, at the level indicated, may be positively detrimental to as well as negatively inadequate for the organism. Whether this suggested deleterious action is a characteristic of gelatin alone or of other proteins also when taken in excessive amounts (*cf.* Newburgh and associates, 1925) remains to be proved. It should be recalled, however, that Osborne and Mendel (1924) have secured excellent growth, without renal changes other than hypertrophy, with diets containing 95 per cent of protein.

Growth upon Diets of Hydrolyzed Gelatin Supplemented with Three Amino Acids.—In a further attempt to arrive at an explanation for nutritive failure of animals upon gelatin diets, we have reinvestigated the influence of complete hydrolysis of the protein preliminary to its incorporation in the ration. The method of hydrolysis has already been outlined. Two diets were employed. Diet E contained 35 per cent of hydrolyzed gelatin and was exactly analogous in composition to Diet D. Diet I contained 15 per cent of the hydrolyzed protein and is comparable to Diet H. After the usual preliminary periods upon Diet A, three rats were transferred to Diet E and four to Diet I. The results are shown in Chart V. As will be observed, the change from the control to the hydrolyzed gelatin rations was followed by the usual losses in weight. After 12 to 16 days upon Diet E, and 4 to 12 days upon Diet I, recovery appeared to occur, and was succeeded by slow growth until the experiments were discontinued. At the expiration of 80 days upon Diet E, Rat 195 weighed 1 gm. less than when transferred to this ration. The net gains of Rats 192 and 194, upon the same diet, were 10 and 12 gm. respectively.

The four rats upon Diet I made somewhat better net gains. However, this difference is due primarily to the fact that, like the animals upon the unhydrolyzed protein, those which received the higher level of hydrolyzed gelatin usually experienced the greater initial losses in weight.

Although no mortality occurred among the seven animals upon these diets (a fact which may or may not be of significance in connection with the suggested toxicity of unhydrolyzed gelatin), nevertheless the growth curves indicate that hydrolysis occasions very little if any improvement in the growth-promoting properties of this protein (*cf.* Charts I and II with Chart V). It appears

that the inadequacy of gelatin as a source of nitrogen is not to be ascribed to the presence of amino acid linkages which cannot be severed by the digestive fluids of the gastrointestinal tract. In

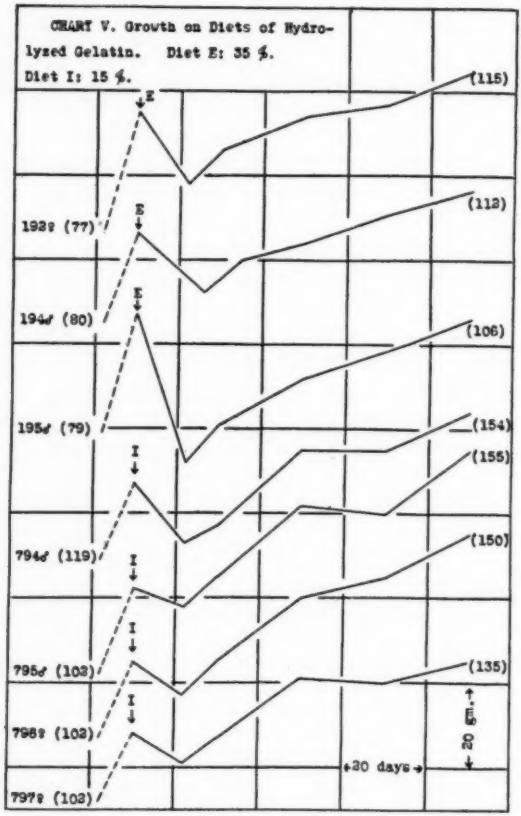

CHART V.

this respect our results are not in accord with those of Totani (1916).

Food Consumption of the Experimental Animals.—In Table III are presented the values representing the average daily food con-

sumption and average daily change in body weight of each rat. For convenience these data are arranged to correspond to the charts.

TABLE III.

Food Consumption and Body Weight Changes of the Experimental Animals.

Chart No.	Rat No.	Diet.	Average daily change in body weight.	Average daily food consumption.	Chart No.	Rat No.	Diet.	Average daily change in body weight.	Average daily food consumption.
			gm.	gm.				gm.	gm.
I	138	A	+2.8	7.8	IV	145	A	+2.3	8.7
		D	−1.9	3.5			B	+0.8	7.0
	139	A	+2.2	7.8			A	+1.5	9.0
		D	0	5.3		151	"	+1.9	5.7
	140	A	+2.5	7.0			B	+1.4	5.4
		F	+0.4	6.7			A	+1.7	8.0
		D	+0.2	6.3		180	"	+3.3	7.6
	150	A	+2.7	5.9			C	+1.1	6.6
		D	−2.3	3.5		182	A	+2.8	6.4
	169	A	+4.9	10.0			C	+1.3	6.0
		D	0	5.1		188	A	+2.8	7.5
							C	+0.8	5.6
II	925	A	+1.7	5.9					
		D	+1.2	6.0	V	192	A	+3.5	7.3
	926	A	+1.8	6.9			E	+0.1	4.2
		D	−1.1	3.1		194	A	+2.6	6.0
	927	A	+2.0	6.7			E	+0.1	3.7
		D	+0.4	6.2		195	A	+3.5	7.9
	928	A	+2.0	6.0			E	0	3.8
		D	+1.5	6.5		794	A	+2.3	7.1
							I	+0.2	8.1
III	141	A	+2.8	8.5		795	A	+2.5	8.3
		F	+0.1	6.0			I	+0.4	7.6
		H	+0.3	5.6		796	A	+2.3	8.3
	798	A	+1.9	6.4			I	+0.4	7.9
		H	+0.3	7.4		797	A	+2.0	8.3
	800	A	+2.3	6.5			I	+0.2	7.5
		H	+0.4	7.2					
	802	A	+2.0	6.8					
		H	+0.3	7.3					

While admittedly the interpretation of food intakes is sometimes a difficult question, we believe the figures recorded in Table III indicate that the *amount* of food consumed by the individual

animals was not the factor which *determined* whether an increase in weight did or did not occur. It cannot be denied that in some of the experiments a rough correlation between food ingestion and rate of growth appears to exist (*cf.* Rats 138, 150, and 926). On the other hand, with many of the animals the intake upon the gelatin diets was almost as large as upon the casein ration, and in some cases even larger. Rat 140 consumed an average of 7.0 gm. of Diet A daily, and 6.7 and 6.3 gm. respectively of Diets F and D. Thus decreases of approximately 4 and 10 per cent in food ingestion were accompanied by decreases of 84 and 92 per cent in growth rates. Rat 927, with a decrease of a little more than 7 per cent in food intake, showed an 80 per cent diminution in growth rate. Other animals, particularly those upon the lower levels of gelatin (Rats 794, 798, 800, 802), ingested just as much of the experimental rations as of the control diet. Both our experience and the observations of others with food mixtures highly deficient in one or more amino acids have taught us to expect very pronounced failure in appetite when the diets are wanting in some factor or factors essential for the growth process. The high level at which many of our animals consumed the gelatin rations leads us to believe that the supplemented mixtures employed in this investigation were not inadequate *to a pronounced degree* in any necessary amino acid. Probably the diminished food intakes of some of the rats upon the diets containing 35 per cent of gelatin may be attributed to the renal involvement.

It should be emphasized also that average figures, such as are recorded in Table III, may lead to erroneous conclusions in that they do not indicate the *progress* of the decreases in food consumption. This point is of considerable importance in arriving at a correct interpretation. An illustration will serve to make this fact clear. Rat 150, one of those in which the failure in growth might be attributed to the diminished food intake, showed an average daily food consumption of 5.9 gm. on Diet A, and 3.5 gm. upon Diet D (*cf.* Table III). Upon the latter ration the average daily loss in weight (2.3 gm.) was greater than was experienced by any of the other animals. Our records indicate, however, that *during the first 4 days upon Diet D, the daily food ingestion averaged 7.2 gm., or was considerably larger than upon the normal casein diet. During the same 4 day period the animal lost an average*

of 1.5 gm. daily. In the three subsequent 4 day periods preceding death, the average daily food consumption amounted to 4.2, 1.8, and 0.8 gm. respectively, accompanied by an increasingly rapid decrease in body weight. In other words, as was observed several years ago by Hopkins (1912) in deficiency studies, the failure in appetite *followed* the failure in growth. For reasons already stated, we are inclined to the belief that renal involvement rather than an amino acid deficiency was the immediate cause of death in many of our experiments, including that upon Rat 150. But whatever may have occasioned the fatal outcome, certainly the initial loss in weight of Rat 150 upon Diet D was not due to insufficient ingestion of food. In a later communication we shall present additional information concerning the relation of food composition to appetite.

SUMMARY.

The above results indicate that diets containing gelatin as the sole protein, at levels of 15 to 55 per cent, are not suitable sources of nitrogen even when supplemented with the amino acids known to be missing, or present in relatively small amounts. About half of the total number of rats placed upon a 35 per cent gelatin diet to which had been added appropriate quantities of cystine, tyrosine, and tryptophane, successfully weathered the change; and after periods of more or less profound "adjustment," maintained body weight or even grew at varying rates.

Reducing the level of gelatin from 35 per cent to 15 per cent of the diet, and incorporating the eleven amino acids which, on the basis of the best available analyses, seem to be the most likely possible sources of deficiency, failed to render the food more satisfactory for growth. It appears that valine and isoleucine, which presumably are absent from gelatin, are not limiting factors in the nutritive properties of this protein. Nor did the increased proportions of phenylalanine, aspartic acid, glutamic acid, and histidine in the supplemented 15 per cent gelatin ration contribute beneficially to the nutritive condition of the animals.

Attempts to improve the quality of gelatin are complicated by what appears to be a deleterious action exerted by the protein when supplied at a 35 per cent level, as manifested by the early fatal outcome of many of the experiments, and the frequent

incidence of severe renal injury. A temporary inhibition in the growth rate of rats upon casein diets may be induced by substituting gelatin for 35 per cent of the carbohydrate.

Diets containing hydrolyzed gelatin supplemented with amino acids do not support growth more successfully than do similar rations containing unhydrolyzed gelatin. Evidently, the peculiar inadequacies of this material for growth purposes are not to be ascribed to the presence in the molecule of linkages which cannot by severed by the digestive mechanism.

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ON THE ISOELECTRIC PRECIPITATION OF PEPSIN. II.

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In a previous paper (1), it was shown that pepsin of high proteolytic power can be obtained by isoelectric precipitation provided the materials are handled rapidly and at low temperatures. Fractions were obtained at pH values ranging from 2.4 to 3.85. The fraction precipitating out at pH 2.4 to 2.5 was shown to have the highest proteolytic activity. This work is an attempt further to purify pepsin by introducing several refinements into the method and to establish the chemical relationship between the various fractions which are obtained. A long series of experiments was carried out during the past year with different solvents, *etc.*, in an attempt to increase the proteolytic properties of the enzyme fractions. We were not able, however, to separate a single sample of pepsin which was superior in digestive power to those already reported. To our knowledge the proteolytic activity of the pH 2.4 to 2.5 fraction is the highest reported by any investigator when assayed by an official and recognized method. We feel that we are dealing with the enzyme in the form in which it naturally occurs in the glandular mucosa of the stomach.

Method of Preparation and Purification.

The method of Fenger and Andrew (1) was employed for the separation and purification of the pepsin. The collection of the stomach linings was started in October and continued through December. These were handled as previously described, and precipitates were collected at pH values of 2.5, 3.0, and 3.8. In order further to purify the fractions and to remove all traces of

ash, they were repeatedly washed in dilute solutions of hydrochloric acid at a hydrogen ion concentration corresponding to the pH value at which the fraction was collected. Practically all impurities should be removed by this process and a purer product obtained than when this procedure is omitted. This step is similar to that used by Field (2) in the preparation of ash-free gelatin. An ample supply of each fraction was prepared.

Properties of the Various Fractions.

All three fractions consisted of white, granular powder, nearly insoluble in distilled water but slowly and completely soluble in acidified water. Determinations of the ash content showed the fractions precipitated at pH 2.5 to be ash-free, while the other two fractions separated at pH 3.0 and pH 3.8 contained 0.025 and 0.010 per cent of ash respectively. The ash determinations were made on 1 gm. samples. The powdered pepsin was placed in platinum crucibles and heated in an electric muffle at temperatures just below dull red heat (500°) in order to prevent volatilization of inorganic chlorides.

The proteolytic activity as determined by the United States Pharmacopœia assay (3) was as follows:

pH	Maximum.	Minimum.
2.5	1:65,000	1:50,000
3.0	1:50,000	1:42,500
3.8	1:45,000	1:40,000

The highest testing fraction is consistently obtained at pH 2.5, confirming our previous results. Forbes (4) likewise found the isoelectric point of two samples of purified pepsin to lie at 2.5 on the pH scale. The existence of pepsin fractions of high proteolytic activity and precipitating at higher pH values is, however, evidenced by the above.

With the view of determining the chemical difference existing between these three fractions, complete analyses were made of composite samples of the three precipitates. The results given in Table I were obtained.

The carbon and hydrogen were determined by combustion, the nitrogen by the Gunning-Kjeldahl method, and the phosphorus weighed as magnesium pyrophosphate. Sulfur and chlorine were determined by the method of Carius.

From the tabulated data it will be seen that some striking differences exist in the composition of the three fractions. The absence of chlorine in the pH 2.5 fraction is significant. The chlorine in the pH 3.0 and 3.8 fractions is, of course, organically bound. The percentage of phosphorus increases with the proteolytic activity of the various fractions, while the percentage of

TABLE I.

	Enzyme fraction precipitated at:		
	pH 2.5.	pH 3.0.	pH 3.8.
	per cent	*per cent*	*per cent*
Carbon	53.15	52.27	51.49
Hydrogen	7.20	6.93	7.03
Nitrogen	14.43	14.62	14.43
Sulfur	0.88	0.96	1.08
Phosphorus	0.13	0.11	0.08
Chlorine	0	0.96	1.02

TABLE II.

	Enzyme fraction precipitated at:					
	pH 2.5.		pH 3.0.		pH 3.8.	
	Nitrogen.		Nitrogen.		Nitrogen.	
	mg.	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
Total N	430.25	100.00	438.62	100.00	280.68	100.00
Amide "	42.95	9.98	38.46	8.77	26.10	9.29
Humin "	26.10	6.06	23.83	5.44	17.16	6.12
Arginine N	8.97	2.05	8.12	1.86	7.85	2.79
Histidine "	11.13	2.59	12.61	2.89	12.12	4.32
Lycine N	10.30	2.38	6.93	1.35	6.07	2.22
Non-amino N	15.31	3.56	22.81	5.20	15.38	5.47
Monoamino "	304.85	70.85	308.46	70.08	189.66	67.53

sulfur decreases. Both these elements are part of the enzyme molecule. This is contrary to Pekelharing and Ringer's (5) contention that the phosphorus is an impurity.

In order to establish further relationship between the fractions the nitrogen distribution was determined by the method of Van Slyke (6).

The results are shown in Table II.

All three samples are high in monoamino nitrogen, and low in

histidine, arginine, and lycine. The sample precipitating at pH 3.8 is higher in arginine and histidine than the other two samples. The non-amino nitrogen is lowest in the pH 2.5 and highest in the pH 3.8 sample, while in the case of the monoamino nitrogen the reverse holds true. In general it may be said, however, that no striking differences occur in the nitrogen distribution of the three samples save for the above exceptions. All three samples reacted positively to Millon's reagent, the xanthroproteic test, and gave strong biuret reactions.

All these analyses are typical of a protein. The first fraction precipitated at pH 2.5 evidently is the enzyme protein, while the pH 3.0 and 3.8 fractions represent early stages of peptic decomposition with progressively decreasing proteolytic activities. Subsequent enzyme fractions remaining in solution at pH values above 3.8 have lost their identity to such an extent that they no longer are able to become isoelectric. These fractions still possess proteolytic properties until they reach the stage when they are sufficiently small to diffuse through parchment or animal membranes. The gradual decrease of proteolytic activity of the enzyme itself is parallelled by loss of its complex protein characteristics.

CONCLUSIONS.

An ash-free pepsin has been obtained with an isoelectric point of 2.5 on the pH scale, and pepsin containing a slight amount of ash has been precipitated at pH 3.0 and 3.8.

Complete analyses have been made of these pepsins and their digestive power determined. The proteolytic activity decreases with increase in pH at the isoelectric point. The analyses in all three cases are typical of a protein.

The authors desire to express their gratitude to Dr. J. J. Vollertsen for valuable assistance and helpful cooperation in carrying out this investigation.

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THE ALKALINE DECOMPOSITION OF CYSTINE.

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For many years it has been customary to classify biological sulfur into "easily split sulfur" and a form which was less easily split. The basis for this distinction has been the test in which the sulfur-containing compound is boiled with lead acetate in an excess of alkali. Under these conditions a part of the sulfur quickly splits off as $S^=$ and, reacting with the $PbO_2^=$, precipitates lead sulfide. Thus the amount of lead sulfide produced was regarded as a measure of the proportion of easily split sulfur present. This reaction has been of special interest because of the conclusions to which it has led concerning the state of sulfur in the protein molecule. Various investigators have obtained from 52 to 83 per cent of the sulfur of cystine as lead sulfide while the proportion obtained from different proteins has varied from about 10 to nearly 100 per cent. A good bibliography of the earlier work in this field has already been published by Hoffman and Gortner (1).

A perusal of this literature shows that slight variations in the conditions of the test have resulted in wide differences in the yield of lead sulfide obtained from even cystine itself, while the more recent studies of Bergmann and Stather (2), Brand and Sandberg (3), and others have demonstrated the great differences in stability of various biological sulfur compounds as regards this reaction.

Inspection of the data obtained by different investigators on the yield of lead sulfide from cystine reveals one very suggestive point: The different percentages reported are nearly all very close to small whole number ratios which suggest their origin from some definite reaction. For example, we have 83 per cent (5:1), 68 per cent (2:1), and 53 per cent (1:1). This last figure, by Schulz (4), resulted from an attempt to raise and reconcile the figures of other workers. He argued that the long heating with alkali afforded too much opportunity for oxidation of the sulfur and to avoid this,

he added metallic zinc to the alkaline solution. The result was a *decrease* in the yield to a little over 50 per cent. A plausible explanation of this ratio would seem to be that the addition of zinc reduced the cystine, in great part, to cysteine and that the latter reacted in the alkaline lead solution to form the lead salt as follows:

$$2\ RSH + Pb^{++} + 2\ OH^- \rightleftarrows (RS)_2Pb + 2\ H_2O$$

which on being heated decomposes as follows:

$$(RS)_2Pb \rightarrow PbS + R_2S$$

This reaction, well known for other mercaptans (Klason (5)) explains the 1:1 partition of the sulfur, while the figure of 53 per cent, as opposed to 50 per cent, is no doubt the result of incomplete reduction by the zinc.

The complicated nature of the alkaline decomposition of cystine is evidenced by the variety of changes involved: The optical activity of the cystine decreases rapidly, sulfide ion is formed, and, in presence of plumbite precipitates as lead sulfide, cysteine is formed, and ammonia is evolved. It is the purpose of this paper to record the results of investigations of some of the various reactions involved.

Qualitatively speaking, the effect of the alkali is to cause gradual decomposition of the cystine with formation of sulfide ions from a part of the sulfur of the decomposed portion. The sulfide is of course, in the presence of the plumbite, precipitated as lead sulfide, but in the absence of plumbite it accumulates and causes reduction of some of the undecomposed cystine to cysteine. The free sulfur resulting reacts with some of the unoxidized sulfide to form polysulfide, and as such, gradually reacts with the alkali in the usual way.

The general practice of heating the cystine for some hours in the solution of alkaline plumbite accelerates these reactions to the extent of making an individual scrutiny of them impossible. Most of the present studies were carried out at about 25°. The lower temperature makes it possible to follow more completely the course of the individual reactions.

EXPERIMENTAL.

Yield of Lead Sulfide.

Some preliminary experiments were carried out to determine such points as the effect of air exposure and time of contact of plumbite on the yield of lead sulfide. The following is typical of

the results obtained. Into each of four Erlenmeyer flasks (Flasks A, B, C, and D) were inserted 100 cc. of 4.00 M NaOH and 1.000 gm. of pure cystine. To Flasks A and C were added at once 10 gm. of $(CH_3COO)_2 Pb \cdot 3 H_2O$ and all four flasks were allowed to stand at room temperature for 24 hours with Flasks A and B slowly swept with pure nitrogen and Flasks C and D exposed to the air. At the end of the 24 hours, the same amount of lead acetate was added to Flasks B and D, all were allowed to stand 1 hour longer, after which the lead sulfide from each was filtered on a weighed Gooch crucible, washed with water and alcohol, and dried in a desiccator to constant weight. Table I shows the results.

While the filtrates from all four flasks after the first filtration were perfectly clear, those from Flasks A and C (lead added at

TABLE I.

Yield of PbS Obtained from Alkaline Cystine Solutions Containing Lead.

After standing:	1 day.		5 days.		11 days.	
Flasks.	Weight of PbS.	Per cent of S as PbS.	Total weight of PbS.	Total per cent S as PbS.	Total weight of PbS.	Total per cent S as PbS.
	gm.		*gm.*		*gm.*	
A	0.3738	18.8	1.3953	70.1	1.5231	76.5
B	0.1019	5.1	1.4098	70.8	1.5004	75.3
C	0.3215	16.1	1.0814	54.3	No further precipitate.	
D	0.0804	4.0	1.3826	69.4	1.4101	70.8

once) were practically colorless while those from Flasks B and D were quite yellow. Evidently the loss of lead sulfide in these cases is to be ascribed to oxidation of the sulfide to polysulfide. Such solutions when acidified always gave a heavy precipitate of free sulfur. On standing these filtrates darkened within 15 minutes at room temperature. They were then allowed to stand 4 days longer under the same conditions of exposure to air as before and the additional lead sulfide was again determined (see Table I, 5 day series). The same process was repeated after 6 days more (a total of 11 days). After this, only negligible amounts of lead sulfide were formed. The failure of Flask C to continue to produce lead sulfide in this series is yet to be explained. In other similar experiments the lead sulfide produced under such condi-

tions attained a total figure between that of Flasks B and D. Otherwise the experiment shows the effect of atmospheric oxidation in reducing the yield as well as the necessity of keeping the plumbite in constant contact with the solution in order to remove the sulfide ion as fast as it is formed and before it can be reoxidized.

A similar series which partly duplicates some of the conditions in the above and also furnishes some information as to the effect of reacidifying before removing lead sulfide is shown in Table II. Four flasks were employed. Flasks, A, B, and C were continually swept with nitrogen while Flask D was exposed to air. The proportions of cystine, alkali, and lead acetate duplicated those in Table I. Flask A was treated exactly as was Flask A in Table I.

TABLE II.

Yield of PbS Obtained from Alkaline Cystine Solutions Containing Lead. 14 Days Standing.

Flask.	Weight of PbS.	Per cent S as PbS.
	gm.	
A	1.5716	78.9
B	1.5420	77.4
C	0.1660	8.3
D	1.5320	76.9

Flask B was treated the same except that the solution was acidified with 100 cc. of 8 M acetic acid just before filtering. Flask C was treated with the lead acetate only 1 hour before filtering the lead sulfide (similar to Flask B in Table I). Flask D was treated as was Flask C in Table I; the lead acetate was added at once and full exposure to air was allowed. All four flasks stood at room temperature for 14 days.

The results, in general, confirm those of Table I. It is evident that acidifying the solution, even though it increases the concentration of Pb^{++}, does not result in any increase in the yield of lead sulfide. As in Table I, the necessity of having the plumbite constantly in the solution is evidenced while with Flask D a figure was obtained of an order which should have resulted in Flask C, Table I.

Potentials of Plumbite Solutions.

As shown above, a high yield of lead sulfide requires the presence of plumbite during the whole time of decomposition of the cystine. Since this supports the transitory character of the sulfide ion it becomes of importance to know something of the maximum level of concentration which sulfide ion can attain in the presence of various concentrations of alkali and plumbite. The usual value for the solubility product of lead sulfide being assumed to hold here ($K = 4 \times 10^{-28}$) the sulfide ion concentration depends on the concentration of Pb^{++} furnished by the equilibrium:

$$PbO_2^{=} + 2\,H^+ \rightleftarrows Pb^{++} + 2\,OH^-$$

TABLE III.

Potentials Obtained from Half-Cells of Lead in Sodium Hydroxide-Sodium Plumbite Solutions with Corresponding Concentrations of Pb^{++} and $S^=$.

Normality of NaOH.	Potential of Pb cell volts.	Concentration of Pb^{++}.	Corresponding concentration of $S^=$.
N			
4.00	0.3129	8.1×10^{-16}	5.0×10^{-13}
2.00	0.2911	4.4×10^{-15}	9.1×10^{-14}
1.00	0.2690	2.5×10^{-14}	1.6×10^{-14}

To determine this, potentials were measured between metallic lead and alkali of various concentrations, saturated with lead hydroxide. Saturated calomel half-cells were used with an absolute potential (at 25°) of +0.5234 volts. The formula

$$E = 0.0295 \log \frac{C}{K}$$

was employed. E = the potential of the lead half-cell, K the solution tension of metallic lead (3.3×10^{-5}) and C the concentration of Pb^{++}. Table III shows the average results obtained.

The figures in Table III show the order of completeness of sulfide ion removal from such solutions. Even when the lack of correction for boundary potentials in the cell and the uncertainty of the solubility product of lead sulfide in such concentrated solutions are admitted, there seems to be no doubt as to the quantitative removal of sulfide when excess plumbite is present.

Optical Activity.

The optical activity of alkaline cystine solutions may be used both as a criterion of the amount of racemization caused by the alkali and also of the amount of reduction of cysteine. While strong alkali has its effect in racemizing cystine, a fair proportion can be recovered with its optical activity practically unchanged, particularly when the solution has not been heated. For example, a solution of 1.5 gm. of cystine ($[\alpha]_D = -207.0°$) per 100 cc. of 4.0 M NaOH stood for 20 days under nitrogen at room temperature. At the end of that time about 0.2 gm. of cystine was recovered, which, under the microscope, was seen to contain some crystals of racemic cystine but which had a specific rotation of $-200°$. However, this figure does not represent the true proportion of *l*- to racemic cystine since the purification process removes a larger proportion of the racemic than of the *l* form (6).

Table IV shows the effect of strong alkali on the rotation of a sample of cystine $[\alpha]_D = -207.0°$. The solution used contained 10.00 gm. of cystine in 500 cc. of 4.0 M NaOH. This solution was allowed to stand at room temperature under pure nitrogen and at intervals 25 cc. aliquots were measured into 50 cc. volumetric flasks and made up to volume with 6.0 M HCl. The result was a solution corresponding to 1.00 gm. of cystine per 100 cc. in 1.0 M HCl and containing also 2.0 M NaCl. The change in conditions, had there been no racemization or reduction, would, because of the sodium chloride, have lowered the $[\alpha]_D$ value to about $-191°$ (7). After cooling, this solution was adjusted to the mark and filtered into a 4 dm. polariscope tube. Rotations were taken at 29°.

After the last aliquots described in Table IV were removed, further aliquots were aerated before rotations were taken. The result was a rise in $[\alpha]_D$ to values as high as $-90°$. It is evident that the change of cystine to cysteine is responsible for a part of the decrease in activity.

This conclusion was confirmed by a number of other experiments. For example, the decrease in activity is markedly accelerated when the above described experiment was duplicated with the addition of sodium sulfide to the cystine solution. The specific rotation, taken at once, showed a drop to about $-50°$ while the sample on being brought to about pH 8 and treated for some hours with a stream of oxygen rose to over $-100°$. Subse-

quent samples gave similar results. In such an experiment the greater proportion of cysteine formed made the rotation much more sensitive to contact with slight amounts of oxygen. Acidification of the samples also gave very heavy sulfur precipitates from the polysulfide formed and complete removal of the colloidal sulfur was necessary before rotations could be read.

On the other hand, if the concentration of sulfide be kept low by the presence of excess plumbite the loss in optical activity is again accelerated but here the loss is due to practically complete decomposition of the cystine. Owing to the presence of less cysteine when plumbite is present (see below), reoxidation produces little or no activity.

TABLE IV.

Specific Rotation of an Alkaline Solution of Cystine. Room Temperature.

Time.	Specific rotation.	Time.	Specific rotation.
hrs.	*degrees*	*hrs.*	*degrees*
0	−191	6	−131.8
½	−181.2	7	−128.0
1	−161.5	23	−88.0
2	−151.0	27	−81.0
3	−142.5	31	−76.5
4	−138.0	47	−51.5

For example, in Table II, the filtrates from all four flasks were examined polariscopically. Those from Flasks A, B, and D were completely inactive. Flask C showed an observed rotation in a 4 dm. tube of −3.04°. For this, calculated to the original 1.00 gm. of cystine, $[\alpha]_D = -76.0°$.

In several experiments (see below) on the rate of loss of cystine and formation of sulfide, polariscopic examinations were made of the unoxidized filtrate from the hydrogen sulfide determinations. For example, a solution of 1.5 gm. of cystine ($[\alpha]_D = -207°$) per 100 cc. of 4.0 M NaOH, after standing in the presence of excess plumbite at room temperature for 3 weeks gave an optically inactive filtrate. Duplication of these conditions but without the plumbite gave a solution with an observed rotation of −0.91° which, on aeration of the solution was raised to −1.22° (both figures calculated to the original concentration of the solution).

Calculated to an original concentration of 1.5 gm. of cystine per 100 cc. (regardless of the increased concentration indicated by Folin-Looney figures) this indicates that about 90 per cent of the cystine had been racemized. Several other similar experiments gave figures of the same order for the degree of racemization. In the absence of any other information it is to be assumed that racemization is proportionally distributed between the oxidized and the reduced forms.

Concentration of Cystine and Cysteine.

In order to follow the changes in cystine and cysteine concentration several series of experiments were made in which samples of pure cystine dissolved in sodium hydroxide were preserved for some months at room temperature and aliquots were withdrawn at intervals for analysis. A number of preliminary trials had demonstrated the important influence of atmospheric oxygen on the course of the reaction. The sulfide formed from the cystine is susceptible to oxidation, if allowed to accumulate in the absence of lead, while the cysteine formed is, in alkaline solution, very much more unstable and easily oxidized. The solutions of cystine in sodium hydroxide were therefore made up under nitrogen. They were preserved in Erlenmeyer flasks equipped with inlet and outlet tubes and were overlaid with a thick layer of paraffin oil. The space above the oil was swept with pure nitrogen and the flasks sealed. The withdrawal of samples was always carried out under nitrogen and after each withdrawal the flask was thoroughly swept again.

The stream of nitrogen was purified, first by passage through alkaline pyrogallol and then through a long silica tube filled with copper shavings and heated. A wash bottle of reduced indigo carmine solution, kept constantly in the train, showed no evidence that any oxygen escaped removal.

The determination of cystine and cysteine was made by the Folin-Looney procedure. Since complete removal of sulfide was necessary before the Folin-Looney reagent could be applied the following procedure was used.

A 250 cc. volumetric flask with short neck, containing the necessary excess of hydrochloric acid, was placed in a vacuum desiccator. From the lid of the desiccator there dipped into the

flask a capillary glass tube connected to the supply of pure nitrogen. The desiccator was then evacuated while a very small stream of nitrogen was carried through the flask. The low pressure inside the desiccator made very efficient sweeping possible. When the flask had been thoroughly swept, a 5 cc. aliquot of the alkaline cystine solution was quickly introduced under the acid and the sweeping continued until all sulfide was removed. Tests on both alkaline cystine and sodium sulfide solutions showed that 2 hours sweeping was ample to reduce the H_2S below any amount which could reduce the Folin-Looney reagent. When sweeping was complete the solution was made up to volume and aliquots for cystine and cysteine were removed and run by the usual Folin-Looney procedure (8).

As indicated by the data in Table V, the concentration of alkali used was varied from 0.1 M to 4.0 M and for each concentration, series were run both with and without lead. In absence of lead, the determination of cystine and cysteine was accompanied by the determination of hydrogen sulfide. The results of the H_2S determinations are also included in Table V and are discussed below under "Production of Sulfide." Cystine of $[\alpha]_D = -209.0°$ was used.

It is evident that in the removal of cystine by the alkali the concentration of the latter plays a very minor part in comparison to the effect of the presence or absence of lead. It is also to be noted that in the 4.0 M NaOH series without lead the apparently very rapid rise in cysteine concentration, as indicated by the Folin-Looney method, brings the sum of the cystine + cysteine to considerably above the amount of cystine originally added to the solution. Also, the sulfide, which was removed before the Folin-Looney determination was made, corresponds to a further amount of cystine. Such results would suggest that the Folin-Looney reagent responds to some other compound present in the solution. To determine whether or not the optical configuration of a sample of cystine has any effect on its response to the Folin-Looney reagent a series of determinations was run in which pure cystine of $[\alpha]_D = -215.0°$ was compared with pure racemic cystine. No difference in readings could be detected. Changes in configuration cannot furnish the explanation for these variations.

It is to be noted that the chief result of the presence of the lead

TABLE V.

Change in Concentration of Cystine, Cysteine, and Sulfide in Alkaline Solutions.

The results are given in gm. per 100 cc.

Time.	No Pb^{++} present.									Excess of Pb^{++} present.					
	1.5 gm. cystine per 100 cc. 4.0 M NaOH.			1.5 gm. cystine per 100 cc. 1.0 M NaOH.			1.0 gm. cystine per 100 cc. 0.1 M NaOH.			1.5 gm. cystine per 100 cc. 4.0 M NaOH.		1.5 gm. cystine per 100 cc. 1.0 M NaOH.		1.0 gm. cystine per 100 cc. 0.1 M NaOH.	
	Cystine.	Cysteine.	H_2S	Cystine.	Cysteine.	H_2S	Cystine.	Cysteine.	H_2S	Cystine.	Cysteine.	Cystine.	Cysteine.	Cystine.	Cysteine.
days															
0	1.5	0	0	1.5	0	0	1.0	0	0	1.5	0	1.5	0	1.0	0
1	1.35	0.10	0.011									0.90			
2	1.30	0.13	0.0135	1.32	0.03	0.0001									
3										0.42	0.22	0.65	0.04		
4				1.25	0.08	0.0001									
5	1.20	0.21	0.021				1.0	0	0.0004						
7	1.15	0.28	0.023							0.21	0.33	0.45	0.10		
8				1.16	0.11	0.0001								0.15	0
9	1.15	0.33	0.026							0.11	0.44	0.37	0.13		
13	1.10	0.51	0.028												
14				1.05	0.20	0.0015									
15										0.02	0.42	0.33	0.16		
16	1.00	0.61	0.030												
20	1.02	0.70	0.030												
21				1.05	0.33	0.006									
22							0.90	0	0.0004			0.30	0.18		
26	1.01	0.69	0.028											0.08	0

21				1.05	0.33	0.006									
22							0.90	0	0.0004			0.30	0.18		
26	1.01	0.69	0.028											0.08	0
30				0.96	0.45	0.011									
32												0.28	0.19		
33	0.94	0.68	0.026												
36										0.02	0.13				
39												0.27	0.19		
43				0.92	0.50	0.019									
44												0.25	0.21		
49	0.88	0.63	0.015												
51														0.05	0
52							0.78	0	0.0008						
53				0.90	0.54	0.025						0.26	0.22		
57										0.02	0.02				
58	0.85	0.59	0.009												
60				0.88	0.50	0.026						0.25	0.23		
68	0.86	0.56	0.003												
71				0.85	0.52	0.028						0.25	0.23		
82	0.83	0.50	0.0009												
96														0.03	0
97							0.76	Trace.	0.002						
205				0.82	0.40	0.052									
206										0	0.01	0.20	0.30		
328										0	0				
332							0.69	Trace.	0.006						
349	0.65	0.55	0.001				0.67	Trace.	0.012						
486	0	0	0												
487				0.76	0.34	0.071									
492												0.16	0.33		
525							0.65	Trace.	0.013						
531														0	0

is a rapid increase in the speed of removal of cystine and cysteine. The rate of formation of the latter seems to be more a function of the concentration of alkali while with the lead the increase is more rapidly brought to a stop. The greater irregularity of the figures for cystine and cysteine in the early part of the curve with 4.0 M NaOH is probably to be ascribed to greater speed of reaction with small amounts of oxygen accidentally introduced.

To obtain some idea as to the conditions existing in alkaline cystine solutions when enough time had elapsed to approximate a

TABLE VI.

Results of Preserving Alkaline Cystine Solutions Away from Air for 128 Weeks at Room Temperature.

2.00 gm. cystine per 100 cc. in:	Observed rotation.	Observed rotation after 3 days aeration.	Cystine.	Cysteine.	H_2S	$[\alpha]_D$ of recovered cystine.
	degrees	*degrees*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	*degrees*
4.00 M NaOH.....	−0.06	−0.13	0.6	1.0	0.001	None recovered.
2.00 " "	−0.02	−0.02	1.4	0.6	0.082	" "
1.00 " "	−0.02	−0.02	1.0	0.5	0.086	" "
4.00 " NH_4OH.....	−2.58		0.9	0.2	0.034	−202.0
4.00 " NaOH + 10 gm. lead acetate..	0	0	0	0	0.084 gm. SO_2.	None recovered.
2.00 M NaOH + 2 gm. lead acetate..	+0.03	0	0.5	0.4	0.095	" "

condition of equilibrium at room temperature, a series of cystine solutions was prepared as indicated below and preserved in Erlenmeyer flasks for a period of 128 weeks. The solutions were prepared under pure nitrogen, overlaid with a heavy layer of paraffin oil, swept with nitrogen, and tightly stoppered. At the end of the 128 week period each flask was opened and determinations were made of cystine, cysteine, sulfide, and of optical activity. The activity was determined on the acidified solution both at once and after aeration for several days and also on any cystine recovered after neutralization and oxidation. The

observed rotation was determined by pipetting a sample of the solution into excess hydrochloric acid of such concentration as to leave an acidity of about 1.0 M and a cystine concentration of 1.0 gm. per 100 cc. if none had been decomposed. Cystine and cysteine determinations were made by the Folin-Looney method and H_2S was determined as described below.

The results (see Table VI) show an almost complete degree of racemization of what cystine remained in all cases except the ammonium hydroxide solution. In this case about 0.6 gm. of cystine was recovered, which was in characteristic hexagonal plate form and had a rotation of −202.0°. When lead was present

TABLE VII.

Results of Preserving Alkaline Cystine Solutions in Contact with Air for 125 Weeks.

Contents of flask.	Observed rotation.	Cystine.	Cysteine.	SO_2
	degrees	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
2.00 gm. cystine per 100 cc. 4.00 M NaOH...	−0.04	0.08	0.08	0.29
0.5 gm. cystine per 100 cc. 4.00 M NaOH... .	−0.03	0.05	0	0.05
2.00 gm. cystine per 100 cc. 1.00 M NaOH...	0	Trace.	0	0.04
0.5 gm. cystine per 100 cc. 4.00 M NH_4OH..	−1.6	"	0	0.01
2.00 gm. cystine per 100 cc. 1.00 M NaOH + 2 gm. lead acetate.....	0	0	0	0.15
2.00 gm. cystine per 100 cc. 4.00 M NaOH + 8 gm. lead acetate.....	+0.09	0	0	0.16
0.5 gm. cystine per 100 cc. 4.00 M NH_4OH + 2 gm. lead acetate.....	+0.08	0	0	0.004

in excess (4.00 M NaOH + 10.0 gm. of lead acetate) instead of H_2S, SO_2 was evolved. The formation of sulfite, in absence of air appears remarkable. In the other flask containing lead the amount of the latter was insufficient to precipitate all the sulfur. In this case, H_2S was evolved as usual.

A similar series was run in which free access of air was permitted. The flasks were partly protected from unduly rapid evaporation and after standing for about the same length of time as before, the evaporated water was replaced and the same determinations made. These results are recorded in Table VII.

In comparing the data in Tables VI and VII it is evident that

although most of the solutions show little or no optical activity when acidified, the Folin-Looney determination indicates that considerable proportions of racemized cystine and cysteine remain when air has been excluded. Excess of air seems to cause nearly complete disappearance of these amounts particularly in the presence of lead. However, in Table VII, the remarkable case of the solution of cystine in NH_4OH, in absence of any lead, shows the presence of a levorotatory compound which evidently does not reduce the Folin-Looney reagent. Even if no racemization and no reduction occurred, the observed rotation of $-1.6°$ would correspond to a cystine content of 0.195 gm. per 100 cc. A similar situation is indicated in Table VI. The NH_4OH solution of cystine ended with a remarkably high observed rotation as compared with the Folin-Looney results. The data in Table V show the reverse situation: Folin-Looney determinations indicating more cystine than was originally placed in the flask. It is evident that under the present conditions the results of this method cannot be taken as an unequivocal measure of cystine content.

Production of Sulfide.

Data on the production of sulfide were included in Tables V to VII in the preceding section. As noted above, when excess plumbite is present the accumulation of sulfide is controlled by the $Pb^{++} \times S^{=}$ product. In absence of plumbite, sulfide was determined by the following procedure, which consisted in acidifying the sample, blowing the hydrogen sulfide into standard iodine solution, and titrating the excess iodine with standard thiosulfate.

A sample of the alkaline cystine solution was measured into a small gas generating flask provided with a separatory funnel and the necessary inlet and outlet tubes. Before introduction of the sample the flask was supplied with the necessary excess of HCl and connected to a train consisting, successively, of a tube containing an asbestos plug, two flasks in series, each containing a measured excess of standard 0.1 N iodine solution with an added excess of KI, and a flask containing a solution of KI and starch. The addition of KI to each flask reduced the possibility of loss of iodine during the determination while the KI-starch solution in the last flask served to catch any small amounts of iodine carried over and to indicate any such condition by its color. This solution

was then added to the iodine solution in the other two flasks when the final titration of excess iodine was made. It thus furnished the necessary indicator and demonstrated any loss of iodine. At the end of a determination the color of the KI-starch solution was never darker than a faint blue which required but a fraction of a drop of standard thiosulfate to discharge. The plug of asbestos served to prevent entrainment from the first flask. This plug cannot be made of glass wool. Lead glass, commonly used in glass wool manufacture, reacts with H_2S turning dark and removes an appreciable fraction of the small amount concerned. The use of glass wool always gave erratic results. Pure nitrogen was used to sweep the train during the determination. All air was thoroughly swept out before the introduction of the sample. Experiments on sodium sulfide solutions demonstrated that 4 hours sweeping was sufficient to remove at least 95 per cent of the H_2S present.

The data in Table V show that the production of sulfide in absence of lead is highly influenced by the concentration of alkali. The rapid rise and fall of the sulfide concentration in 4.0 M NaOH is not paralleled in any other solution within the length of time employed (see also Table VI). No other of these reactions shows a speed so directly influenced by the concentration of the alkali.

The amount of sulfide determined at any given time is not to be regarded as the total formed up to that time. The equilibrium

$$RSSR + S^{=} + 2\,H_2O \rightleftarrows 2\,RSH + S + 2\,OH^{-}$$

which is responsible for part of the production of cysteine changes part of the sulfide to polysulfide and is undoubtedly responsible for the precipitation of free sulfur when these solutions are acidified.

The production of sulfur dioxide instead of hydrogen sulfide in all cases of atmospheric exposure (see Table VII) is probably of no significance as regards the chemistry of cystine decomposition. In the presence of air and strong alkali the formation of sulfite from sulfide or polysulfide is a common occurrence. Some further oxidation to sulfate was indicated by qualitative tests and various other intermediate oxidation products of sulfur were no doubt present (thiosulfate, *etc.*).

Production of Ammonia.

The fact that certain amino acids, including cystine, evolve ammonia on being heated with sodium hydroxide solution has been known for some years. Van Slyke (9) noted that the amount of ammonia derived from boiling a sample of cystine in alkali under certain specified conditions was about 18 per cent of the total when the boiling was done in glass and was raised to over 69 per cent when the same reaction was carried on in a copper flask. More recently Plimmer and Lowndes (10) in investigating the estimation of cystine in the modified Van Slyke method determined the percentage deaminization of both pure cystine and "hydrolyzed" (racemic) cystine and found a slightly greater loss of ammonia with the racemic sample.

It appeared worth while to determine if any relationship existed between ammonia production and the other reactions described above. An experiment at room temperature showed plainly the marked influence of plumbite. 1.5 gm. of cystine ($[\alpha]_D = -207.0°$) were dissolved in 100 cc. of 1.00 M NaOH, placed in an aerating flask, and slowly swept with pure nitrogen for several days at room temperature into a flask containing standard acid. At intervals the excess acid was titrated and replaced with a fresh supply. The same experiment was duplicated with the addition of 1.7 gm. of lead acetate to the cystine solution. Table VIII contains the results.

Other experiments yielded comparable figures. Conditions being equal, the effect of the lead was to more than double the rate of ammonia production.

A much more elaborate series of experiments was carried out at 100°. The alkaline cystine solutions were not boiled but were placed in a steam-jacketed container and swept with pure nitrogen at a standardized rate of speed for 5 hours. This procedure gave easily duplicable results and was obviously much preferable to direct boiling of the solution.

To control the effect of variation in the flow of nitrogen, two determinations were carried on simultaneously, in series with each other as regards the nitrogen stream and each day the two consisted of a duplicate of the determination of the previous day plus a new one. Thus duplicates were always run on different days in series with different determinations. In all cases 0.2500 gm. of

cystine was dissolved in 50.0 cc. of the alkali used and any heavy metal salt desired was added. Unless otherwise specified C. P. *l*-cystine ($[\alpha]_D = -207°$) was used. The ammonia was swept, as usual, into excess acid and titrated with alizarin. Table IX shows the results obtained.

The data in Table IX show the rather remarkable result that the production of ammonia is *inversely* proportional to the concentration of alkali from 4.00 M to about 0.2 M NaOH. From 0.2 M to 0.001 M solutions the speed of ammonia production drops sharply. In the presence of an amount of lead equivalent to the sulfur present the rate of ammonia evolution is more than tripled. This effect is not the result of catalysis by the lead sulfide formed as

TABLE VIII.

Deaminization of Cystine at Room Temperature.

Time.	Percentage deaminization.	
	Cystine alone.	Cystine + plumbite.
days		
3	2.3	9.2
8	8.1	
10		28.6
13	13.1	
17		41.1
21	20.6	
22		49.1

it was found in other experiments that addition of freshly precipitated lead sulfide to the solution had no effect on the production of ammonia. Moreover, the action is probably not catalytic in nature at all since reduction of the lead to 10 per cent of the theoretical gave a result only slightly higher than that obtained when no lead was present, while doubling the theoretical amount of lead raised the percentage decomposition to 74 per cent.

The figures obtained from copper show a much less marked effect from copper sulfate and no effect at all from the presence of metallic copper in the flask. The effect of ferric chloride was also very slight.

It was presumed that the results with copper and iron were the result of a less effective concentration of these metals since the

TABLE IX.

Deaminization of Cystine at 100°.

Concentration of NaOH.	Heavy metal salts added.	Optical activity of cystine $[\alpha]_D$.	Percentage deamination.
M		*degrees*	
4.00			10.5
			11.2
2.00			18.1
			17.5
			17.8
			17.9
1.00			21.9
			22.1
0.2181			23.8
			23.5
0.100			21.4
			21.9
0.050			19.9
			18.5
0.010			11.7
			10.4
0.001			3.1
			1.3
2.00	0.079 gm. lead acetate (10 per cent theory).		20.5
			19.8
2.00	0.79 " " " (theory).		61.5
			59.6
2.00	1.6 " " "		74.0
			74.4
2.00	0.3 " copper sulfate.		23.3
2.00	0.52 " " " (theory).		50.2
2.00	0.4 " metallic copper.		17.7
			16.7
2.00	0.375 " ferric chloride (theory).		18.7
			18.0
2.00	0.75 " " "		18.5
			18.1
2.00	0.382 " zinc acetate (theory).		19.3
2.00	0.26 " disodium arsenate.		18.4
			20.0
2.00		−148.7	18.1
			18.1
2.00		0	18.3
			18.2
2.00		+9.0	18.4
			18.0
2.00	0.79 " lead acetate (theory).	+9.0	59.5
			59.6

hydroxides of both were precipitated and that of the copper partly dehydrated. Therefore, zinc was selected as an amphoteric metal with a moderately insoluble sulfide for comparison with lead. The results, however, showed practically no increase over the controls.

On the hypothesis that more complete removal of sulfide ion was necessary than was afforded by zinc, a metal was selected which would effect more complete removal. Arsenic sulfide is not only very slightly ionized but it unites with excess alkali to form soluble and very stable sulf-arsenates. Disodium arsenate in proportion equivalent to the sulfur was used but with negative results. It must be concluded that this very marked effect of lead is of highly specific nature.

In order to determine whether optical configuration had any influence on speed of deaminization a series of cystines was used with specific rotations varying from $-207°$ to $+9.0°$. The results indicated that optical configuration had no effect, either in the presence or the absence of plumbite.

One of the most outstanding features of the decomposition of cystine in alkali is the marked and specific effect of lead on the various reactions involved. This is particularly true of the rate of loss of cystine and the rate of deaminization. Although the initial formation of cysteine seems to be very little affected by the lead, the latter causes its decomposition to set in more rapidly than would otherwise be the case. This specificity of lead in cystine chemistry leads to interesting speculations as to any possible connection with its very specific properties as a poison. The evident independence of the various reactions studied emphasizes the complexity of the alkaline decomposition of cystine while the ease with which the production of sulfide can be varied by the conditions employed shows the futility of the old classification of "easily split sulfur" unless conditions are very definitely controlled and described.

SUMMARY.

The decomposition of cystine in alkaline solution has been studied with reference to the rate of some of the reactions involved. There has been determined, chiefly at room temperature, the rate of formation of sulfide and of cysteine and the rate of loss of cystine. The effect on these reactions of variation in concentra-

tion of alkali and of the presence or absence of lead has also been determined. The presence of lead greatly accelerates the decomposition of both cystine and cysteine.

The potentials of sodium plumbite solution with excess sodium hydroxide against the lead electrode were determined and from these was calculated the probable range of Pb^{++} concentration in such solutions.

The speed of racemization of cystine in alkali was measured and found to be much greater than the speed of decomposition.

The rate of deaminization was measured under a variety of conditions. This rate is greatly increased by the presence of amounts of lead sufficient to precipitate all the sulfur as PbS. Copper salts have a much smaller effect and metallic copper none while zinc, iron, and arsenic salts were practically without influence. The specific effect of lead here demonstrated corresponds to its strong influence on the rate of loss of cystine.

The optical configuration of the cystine is without influence on the rate of loss of ammonia.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF LIPOIDAL PHOSPHORUS IN BLOOD.

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The lipoids are extracted from oxalated blood with an alcohol-ether mixture and after the solvent is evaporated on the water bath, the organic matter is destroyed with concentrated sulfuric acid and hydrogen peroxide. The excess acid is neutralized with ammonia and reacidified with acetic acid. The phosphate is then precipitated with uranium acetate and determined colorimetrically by a procedure recently described in this *Journal* (1).

Procedure.

In a 10 cc. volumetric flask place 8 cc. of a mixture of redistilled ethyl alcohol 3 parts and ethyl ether 1 part. While rotating the flask introduce into the mixture 1 cc. of well mixed oxalated blood slowly, drop by drop, so as to prevent the formation of large clumps. Immerse the flask in a large beaker of boiling water with frequent and vigorous shaking until the liquid in the flask just begins to boil. Cool to room temperature by immersing the flask in cold water, and add alcohol-ether mixture to the 10 cc. mark. Filter through an ashless filter paper, covering the funnel with a watch-glass to prevent evaporation.

Introduce 5 cc. of the filtrate (equivalent to 0.5 cc. of blood) into a 15 cc. Pyrex centrifuge tube graduated at 10 cc. Immerse the tube in a beaker of boiling water and evaporate to dryness. Add 0.3 cc. of concentrated H_2SO_4 and heat very gently over a small flame of a micro burner until frothing ceases and an abundant supply of SO_3 fumes is produced. Remove the tube from the flame and by holding it in an almost horizontal position rotate it to bring the acid in contact with the adherent lipoidal material on the walls of the tube. Rotate until the walls are completely covered

with the acid and heat again gently for another minute. Let cool and add 5 drops of 30 per cent hydrogen peroxide until the liquid becomes clear but of a deep red color, and again rotate the tube to moisten the walls as before. Boil again until SO_3 fumes begin to appear. The liquid should now be perfectly clear and colorless. If a yellow tinge should still persist, add another drop of H_2O_2 and again heat until the SO_3 fumes begin to come off. Allow to cool and add 5 cc. of water.

Into a similar test-tube place 5 cc. of standard phosphate solution.

Add to each tube 1 drop of brom-thymol blue indicator solution, and neutralize with dilute ammonia (1 part of 28 per cent ammonia and 4 parts of water) until the solutions just turn blue, and add

TABLE I.

Tube No.	Ammonium sulfate added (50%) cc.	Phosphorus.	Colorimetric readings. Tube 1 used as standard set at 20 mm.
		mg.	*mm.*
1	None.	0.05	19.9
2	0.1	0.05	20.3
3	0.2	0.05	20.2
4	0.5	0.05	19.5
5	1.0	0.05	20.5
6	2.0	0.05	20.1
7	3.0	0.05	19.7

0.3 cc. more of ammonia. Make just acid with 5 per cent acetic acid; add 1 cc. of uranium acetate solution and let stand for a minute. Add 2 cc. of 95 per cent alcohol, shake well, and let stand for 15 minutes. Centrifuge for about 5 minutes at high speed and decant the supernatant fluid, removing the last drop by touching the mouth of the tube against a clean filter paper. Add 5 cc. of 20 per cent alcohol and with a fine glass rod break up the precipitate and add 5 cc. more of alcohol, washing down the rod. Centrifuge again and wash once more with 20 per cent alcohol. Pour off the final wash fluid as completely as possible and dissolve the precipitate in 1 cc. of 20 per cent trichloroacetic acid. Add 5 cc. of water and 1 cc. of a 10 per cent solution of potassium ferrocyanide, and fill up with water to the 10 cc. mark. Mix well and

after 2 minutes compare in the colorimeter, setting the standard at 20 mm.

Calculation of Results.

$$\frac{S}{R} \times 10 = \text{mg. phosphorus in 100 cc. blood.}$$

S = reading of standard in mm.
R = " " unknown " "

DISCUSSION.

Some details of the method, as well as the preparation of reagents, are omitted from this paper, as they have already been described in the previous paper (1).

The choice of a proper solvent for the extraction of lipoids is of great importance. Chloroform and ether do not extract completely all the lipoid material; they also present the disadvantage that the tissue must first be dried before being subjected to extraction by these solvents. Kumagawa and Suto (2) found that hot alcohol gave the largest yield of substances soluble in ether; and as pointed out by Bloor (3), it has the advantages in that it penetrates the tissues readily, and can be applied directly to the moist tissue without previous drying. Various solvents have been tried and that of Bloor (4) with alcohol-ether, was found to give the best results.

While it is well known that the phosphorus content of an alcohol-ether extract is not a true measure of the lipoidal content, since other substances containing phosphorus, not lipoid, are extracted along with the lipoids, yet this is the only means available, since isolation of lipoids in a pure state is a matter of great difficulty, and utterly impossible in small amounts of tissue.

Another matter of importance is the means used for the destruction of the organic matter in order to liberate the phosphorus. Bloor (4) digests the organic matter with sulfuric and nitric acids. Roe, Irish, and Boyd (5) use sulfuric and nitric acids and complete the digestion with H_2O_2. Baumann (6) uses sulfuric acid only and H_2O_2, so do Koch and McMeekin (7), in the digestion of the Folin-Wu filtrate for the determination of non-protein nitrogen in blood. I have found the sulfuric-nitric acid mixture not as satisfactory in the digestion of lipoidal material as the sulfuric acid alone with the H_2O_2. It was found that 0.1 cc. of concen-

trated H_2SO_4 is sufficient to destroy completely all the lipoidal extract from 0.5 cc. of blood. However, 0.3 cc. is used in the method because it shortens greatly the heating time and because this amount of fluid covers more completely the sides of the tube during the process of rotation. This process of wetting the sides of the tube with the acid is very essential, since during the process of evaporation of the alcohol-ether mixture a considerable am unt of lipoidal matter sticks to the walls of the tube, and would otherwise escape digestion, thus incurring a loss of phosphorus. It was pointed out by Baumann (8) that on prolonged heating at a high temperature a loss of phosphorus may occur. However, Roe, Irish, and Boyd (5) found that no loss occurs at a temperature of 200°. No loss of phosphorus occurs in the present method. This was checked with added known amounts of phosphate. The whole process of digestion is done very rapidly, in about 2 minutes, and the heating is done very gently over a small flame, so that no superheating occurs.

The next question of importance was to find out whether the ammonium sulfate produced during the neutralization of the sulfuric acid with ammonia does not interfere with the precipitation of uranium phosphate. As Table I shows, ammonium sulfate in great concentration does not interfere with the precipitation of uranium phosphate.

SUMMARY.

A method is described for the determination of lipoidal phosphorus in small amounts of blood by extracting the lipoidal material with alcohol and ether. The alcohol-ether is evaporated and the organic matter is destroyed with concentrated H_2SO_4 and H_2O_2. The phosphorus is then precipitated as uranium phosphate and determined colorimetrically.

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XUM

OBSERVATIONS BEARING ON THE DETERMINATION OF VITAMIN A.

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About 2 years ago the Protein Laboratory, in cooperation with the Drug Control Laboratory of the Food, Drug and Insecticide Administration, began an investigation of the vitamin content of cod liver oils and products made therefrom. In vitamin A studies we were confronted with certain fundamental considerations of technique.

The United States Pharmacopœia X contains a method for vitamin A assay of cod liver oil. As related by Sherman (1) the technique is essentially that developed by Sherman and Munsell (2), and is based upon the earlier work of Drummond and his associates.

The work of Steenbock and Nelson (3) showed that cessation of growth of rats on a diet deficient in vitamins A and D may be owing to a lack of either one of these factors depending upon the relative amounts stored by the animal before it is put upon the deficient diet. In the Pharmacopœia method proper weight is not given to the growth-promoting influence of vitamin D. Accordingly, under certain conditions the cessation of growth owing to a depletion of the store of this vitamin may be taken as a criterion of the depletion of the animal's store of vitamin A. A previous attempt by one of us to use the Pharmacopœia stock diet met with unsatisfactory results. Within a period of 3 months the condition of the animals indicated nutritive failure. They lost weight, their coats became rough, and reproduction was very poor. By modification of the diet most of the animals were quickly restored to normal condition.

In some of our preliminary work we used a stock diet similar

to that used by Steenbock (5), differing principally in that it contained whole milk powder and no fresh milk. It had the following percentage composition: whole milk powder, 15; yellow corn, 64; commercial casein, 5; linseed oil meal, 13; alfalfa meal, 2; calcium carbonate, 0.5; and sodium chloride, 0.5. This diet proved to be too low in vitamin A for satisfactory results. Many of the females had transient ophthalmia. Reproduction was not satisfactory, and some of the young developed ophthalmia as early as 23 days after being put on a vitamin A-deficient diet, even though they had reached a weight of more than 40 gm. 21 days from birth. The addition of 5 per cent of butter fat to the diet improved it greatly. The corn used was excellent in quality. The alfalfa meal was of poor quality and may not have furnished much vitamin A, but it was surprising that the amount of milk in the diet did not compensate for this deficiency. The milk powder was obtained in 50 pound lots, this quantity being used up in a period of 3 weeks. Possibly the manner in which this material had been handled had been injurious to its vitamin A. We took these observations into consideration in studying the limitations of the Pharmacopœia vitamin A assay of cod liver oil.

We decided to test further the possibility of using the cure of ophthalmia as a quantitative method for vitamin A determination and to study the relationship between such cures and growth response in the animals.

EXPERIMENTAL.

Application of the United States Pharmacopœia Method.

Ten stock female albino rats, primiparas, were divided into two equal groups, Groups A and B, mated, and given the stock diet prescribed by the Pharmacopœia: whole wheat, 33 per cent; whole milk powder, 66 per cent; and sodium chloride, 1 per cent. The males in the two groups were interchanged daily. The milk powder in the diet of Group A was taken from 50 pound tins used for our other stock diets, and for Group B the same brand of milk was taken from 1 pound vacuum cans. Six litters of rats were raised, two from Group A and four from Group B. Two of these litters consisted of six rats each, and the remaining litters were reduced to that number when the young were 7 days old.

We had intended to place all the young rats on a vitamin A-deficient diet at the age and weight prescribed in the Pharmacopœia method, "not less than twenty-five days and not more than twenty-nine days old, and weighing not less than 35 Gm. and not more than 45 Gm.," but our animals were too large to come within those limits. One litter from Group A (Lot 165) and one litter from Group B (Lot 164) were started on a vitamin A-free diet at 21 days of age. The average weights of these animals were 47 and 44 gm. One litter from each of the foregoing groups (Lot 166 and Lot 167) was started at 25 days of age, the animals averaging 53 and 49 gm., respectively. The two remaining litters from Group B were started at 28 and 29 days and averaged 52 and 61 gm., respectively.

The vitamin A-free diet used in all the experiments described in this paper had the following composition:

	per cent
Casein	18
Salt mixture	4
Agar	2
Yeast	8
Dextrin	67
Activated peanut oil	1

In those experiments in which both vitamins A and D were omitted, the activated peanut oil was replaced by an equal weight of dextrin.

The casein was a commercial product extracted ten times with hot 95 per cent alcohol. Casein in small cloth bags was covered with alcohol and kept at a temperature of 60 to 75° for 7 hours in a specially constructed extractor. The alcohol was then drained off and the casein permitted to drain overnight. Fresh alcohol was then added and the extraction repeated. From 30 to 40 pounds of casein were extracted at one time, and from 20 to 25 liters of alcohol were used for each extraction. The extracted casein was freed from alcohol in a steam-heated drier and then ground. The salt mixture was Osborne and Mendel's Salt IV (4). When used as a sole source of vitamin B, and fed at a level of 0.6 gm. per rat per day, the yeast permitted growth at the maximum rate. Dextrin was prepared from corn-starch by autoclaving the moistened starch for 2 hours at 15 pounds pressure, drying in a current of air

at 50–55°, and then grinding. The peanut oil carried 10 per cent of crude cholesterol which had been irradiated with ultra-violet light to about its maximum antirachitic activity. Besides serving as a vehicle to permit uniform distribution of the activated cholesterol, the peanut oil imparts desirable physical characteristics to the diet. It prevents the settling of finer particles and also prevents the inhalation of dustlike particles which may be particularly irritating to mucous membranes of the respiratory tract which are affected by vitamin A deficiency.

The rats were kept in individual cages provided with false screen bottoms to prevent access to excreta. Each litter was divided into two groups of three rats each. One group received the vitamin A-free diet and the other the vitamin A- and vitamin D-free diet. The rats which received vitamin D grew at practically a normal rate until sore eyes became evident, whereas those which received no vitamin D ceased to grow or grew very little after being on the diet 2 weeks. The average maximum weight of the rats on the vitamin A-free diet before showing ophthalmia was 116 gm., ranging from 101 to 135 gm. The corresponding average maximum weight for the vitamin A- and vitamin D-free diet rats was 79 gm., with a range of 68 to 100 gm. The average gain for all the rats receiving vitamin D previous to vitamin A additions was 37 gm. more than the rats which received no vitamin D. In all cases where the rats received vitamin D ophthalmia developed nearly simultaneously in each litter. In only one litter, Lot 165, did sore eyes become evident as early in the rats which received no vitamin D as in those which received that component. The delay in most cases was a few days, but in three instances unmistakable ophthalmia did not develop until from 44 to 50 days.

Four of the litters were given butter fat as a source of vitamin A when ophthalmia had developed. The butter fat additions to the diet were made when the condition of the eyes could be easily recognized as abnormal, but before suppurative conditions had become established. There was marked redness and some swelling of the lids, and in many cases bleeding or slight lacerations due to scratching. Depilation of the lids, quite characteristic of hooded rats in the early stages of xerophthalmia, is not so readily recognized in the albinos. Lots 164 and 165 received 100 mg. of butter fat each daily, and Lots 166 and 167 received 200 mg. The butter

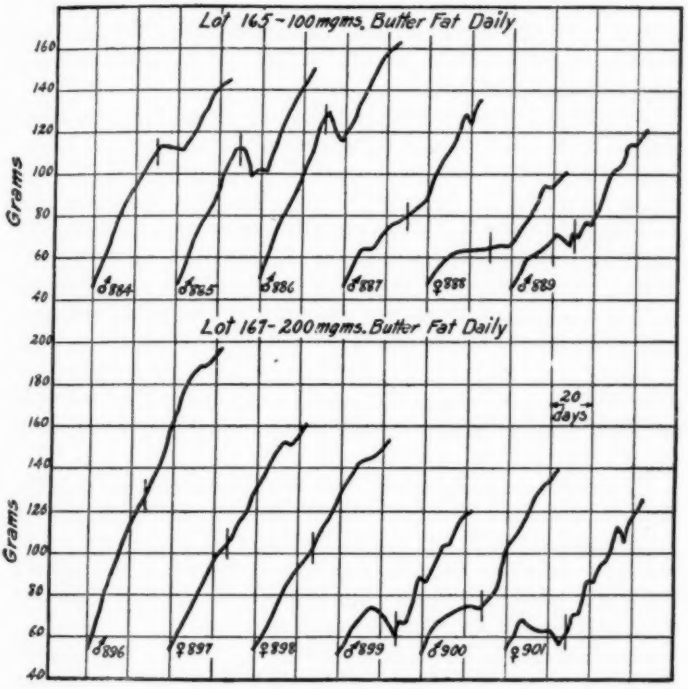

CHART I. The curves show the rate of growth of rats on a vitamin A-deficient diet with and without vitamin D, and the extent of growth induced in 35 days of butter fat feeding after ophthalmia had been produced. The beginning of butter fat feeding is indicated by a short vertical line intersecting the growth curve. The animals in Lot 165 were from one litter and Lot 167 another. The first three rats in each litter received an adequate amount of vitamin D from irradiated cholesterol throughout the experiment. The last three rats in each litter received no vitamin D except that contained in the butter fat. Previous to the butter fat addition the rats which received vitamin D grew much more rapidly than those which did not, but after the butter fat addition the average increase in weight was slightly greater in the rats which had received no vitamin D.

fat was prepared from unsalted butter. It was melted and filtered to remove proteins and water, and stored at a freezing temperature. Each day a portion was brought to a temperature of 60°, and the desired amounts were transferred with a graduated pipette to small glass dishes, and fed.

The growth curves of Lots 165 and 167 shown in Chart I are typical of the performance of the animals in the other two lots. In no case was there a cure of ophthalmia on the 100 mg. level of butter fat up to the end of 35 days, when the experiment was terminated. In general, there was a slow improvement during the first 2 or 3 weeks with little, if any, change after that. On the 200 mg. level there was an early improvement in the eye condition of all the animals and with one exception all the animals became practically free from symptoms of vitamin A deficiency in from 10 to 22 days. In nearly all cases there was a slight recurrence of redness of the lids, usually in one eye, which was easily discernible for intervals of a few days, then disappeared. One rat was never free from ophthalmia.

The average increase in weight for six rats on the vitamin A-free diet which received 100 mg. of butter fat daily was 42 gm., and for those which received 200 mg. of butter fat it was 63 gm. The average weight increase of six rats on the vitamin A- and vitamin D-free diet which received 100 mg. of butter fat was 50 gm., and for those which received 200 mg. it was 60 gm.

The difference in the time of onset of ophthalmia in Groups A and B was not marked, although the young from Group A showed these symptoms an average of 5 days earlier than did rats started from Group B at the same age and nearly the same weight. The data obtained were too limited to furnish conclusions as to the relative merits of using whole milk powder from small evacuated containers or from large containers with no special precautions taken to preserve vitamin A.

Cure of Ophthalmia and Growth as an Index of Vitamin A Potency.

In testing medicinal cod liver oils for the vitamin A potency we have used the cure of ophthalmia as a criterion and at the same time obtained information on the amount of growth induced during the curative period. The rats used in these experiments were taken from our stock colony which received a diet essentially the

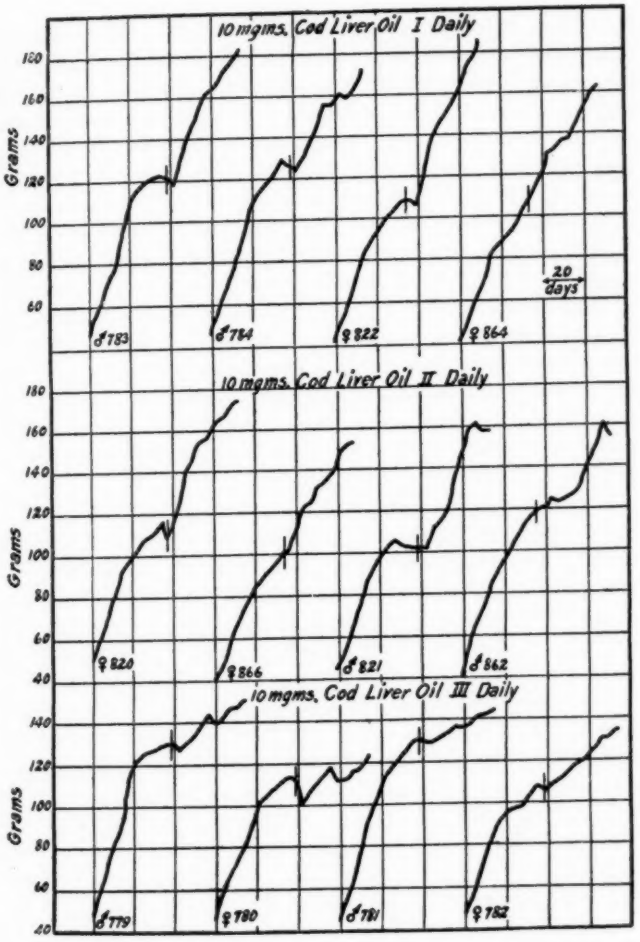

CHART II. These growth curves are typical of the results obtained in testing cod liver oils by the technique described in the text. The vertical lines intersecting the growth curves mark the beginning of cod liver oil feeding. The curative test was begun when ophthalmia was definitely established. The weights of the animals when the curative tests were begun varied from 100 to 130 gm. Only one animal remained stationary or declined in weight for a period of 7 days previous to the time cod liver oil feeding was begun. The average weight increase during the 35 day test period on Cod Liver Oil I was 60 gm.; Cod Liver Oil II, 52 gm.; and Cod Liver Oil III, 19 gm.

same as that used by Steenbock (5). In place of whole milk the animals received whole milk powder incorporated in the diet, and some received butter or carrots in addition. Rats 21 to 23 days old and weighing 40 to 45 gm. were given the vitamin A-free diet previously described until ophthalmia developed. During this preparatory period the rats, in groups of two or three, were kept in cylindrical wire cages 8 inches high and 11 inches in diameter, having raised bottoms of galvanized wire with three meshes to the inch. When the rats showed sore eyes which could be easily recognized, red or slightly swollen and frequently bleeding lids, they were put in similar individual cages and given cod liver oil. Such rats have ranged from 100 to 145 gm. in weight. The cod liver oil was mixed with peanut oil so that the amount to be fed each day would be contained in 0.2 cc. of the mixture. The oil was placed before the animals in small glass dishes. The curative period was limited to 35 days. Four rats from four different litters were used in each test.

The growth curves resulting from one test on each of three cod liver oils are given in Chart II. These results were selected from more than 50 tests carried out on twenty-six cod liver oils as being typical of the performance of our animals. They also represent the range of variation in vitamin A of about 90 per cent of the oils examined. Cod Liver Oil III is the lowest in vitamin A examined to date, and two oils have been found to be distinctly richer in vitamin A than Cod Liver Oil I. There were three rapid cures of ophthalmia on Cod Liver Oil I. Rat 864, whose eyes showed a slightly more advanced stage of ophthalmia when changed, did not show complete absence of the disease during the test period. On Cod Liver Oil II three rats were completely cured of ophthalmia, but not as rapidly as on Cod Liver Oil I. Rat 862 was not completely cured. The condition of the eyes of the rats on Cod Liver Oil III remained practically the same during the test period.

The performance of control rats kept on the vitamin A-free diet simultaneously with the cod liver oil tests is as follows: An average weight of 100 gm. was attained in 20 days, a gain of nearly 3 gm. per day. The growth rate then gradually declined, and a maximum of 120 to 125 gm. was reached between the 40th and 45th day. At this point the range in weight was from 100 to 150 gm., with a marked increase in weights of males over females. Deaths

occurred as early as the 45th day, and one animal lived 85 days. The length of survival was extremely variable, even with animals from the same litter. Symptoms of ophthalmia could usually be detected between the 35th to the 40th day, but there were variations ranging from 30 to 46 days.

DISCUSSION.

The results which we present with respect to the application of the United States Pharmacopœia vitamin A assay of cod liver oil are not to be considered typical of the results to be obtained by this method. They are presented to point out certain defects in the Pharmacopœia assay which the producers of cod liver oil, who are principally interested in this method, have undoubtedly encountered. The Pharmacopœia assay provides in part:

"The rats shall begin receiving the Cod Liver Oil to be tested after not less than seven days of stationary or declining weight, and from the time of feeding the test oil, they shall be kept in separate cages."

"The test period shall continue for thirty-five days and the potency of the oil shall be judged from the rat or rats showing a gain on the thirty-fifth day of between 10 and 20 Gm. over the weight at the beginning of the test, and the cure of the induced symptoms of vitamin A starvation."

It is obvious from Chart I that the stock diet was so low in vitamin D that storage in the young was very low. If the animals in Lot 167 had been changed to the material tested after 7 days of stationary or declining weight, Rat 901 would have been changed after 14 days; Rat 899, 21 days; and Rat 900, 28 days, on the vitamin A- and vitamin D-free diet. The growth of the rats on the vitamin A-free diet shows the fallacy of depending on the growth rate to determine when stored vitamin A is depleted in the animal, if vitamin D is not provided in adequate amounts.

If the Pharmacopœia method is modified so that vitamin D is supplied to the test animals, our experience indicates that other details of that method cannot be followed to advantage. There may be retardation of the growth rate before symptoms of ophthalmia become apparent. If the curative test is not begun until the animal has been at a stationary or declining weight for a period of 7 days, the test must be conducted on an animal that may have reached a critical condition, and that condition will not be greatly improved if the level of vitamin A fed permits but little

growth. (In our method of feeding the oil, results are further complicated by unsatisfactory oil consumption on such low levels.) Furthermore, we do not get uniform cures of mild cases of ophthalmia during the test period unless the animals receive sufficient vitamin A to permit growth at approximately the maximum rate. For that reason the growth requirements in the Pharmacopœia become meaningless if the induced symptoms of vitamin A starvation are cured.

Ophthalmia has occurred in practically 100 per cent of the animals used in our vitamin A tests. Over a period of 2 years only two rats failed to develop detectable symptoms of sore eyes on vitamin A-deficient diets. One of these animals died after being on the experimental diet 35 days, a few days before its litter mates showed ophthalmia. The other rat died at 52 days, 6 days after the other animals in the litter had been put on curative tests. Aside from the fact that the animals we used must have had a comparatively low storage of vitamin A, we attribute our success in developing ophthalmia to the fact that we used only vigorous animals, that grew at a rapid rate on the vitamin A-free diet.

Just what is intended by the statement in the Pharmacopœia that, "The antirachitic requirements of the breeders shall be assured." is not clear to the writers. If it means that reproduction and subsequent growth of the lactating young shall not be influenced by a lack of vitamin D, which seems the most plausible interpretation, then it is quite probable that no vitamin D other than that contained in the prescribed stock diet is necessary. We are not familiar with any studies in which the Pharmacopœia technique has been applied where an attempt has been made to provide the mothers with sufficient vitamin D to enable the young to acquire enough of that vitamin so that the amount of growth on a vitamin A- and vitamin D-free diet will be dependent only on the vitamin A storage. If this can be done successfully, the first symptoms of vitamin A deficiency will be the same as when the test diet is deficient only in vitamin A, and from 10 to 20 mg. per day of most cod liver oils will be required to meet the Pharmacopœia definition for a unit. If the United States Pharmacopœia method had contained a statement to the effect that adequate vitamin D must be provided the test animals at all times, it is very doubtful if cod liver oils would be marketed at the present time with claims

which could be justified of containing 500 or more U. S. P. units of vitamin A.

We believe that the technique we have used in testing cod liver oil will give more trustworthy results than are generally obtained by the Pharmacopœia technique. The rate of growth during the test period is a more tangible manifestation of vitamin A potency than cure of ophthalmia, but data should be obtained on both. Some cases of ophthalmia apparently cured will recur a few days later and again disappear. It therefore becomes difficult to define a cure of ophthalmia. Relative results are not so difficult to obtain, but a definite standard which an oil must meet is not easily arrived at.

Vitamin A can be determined quantitatively by a technique which will eliminate most of the criticisms directed at the Pharmacopœia method. Data have been obtained in this laboratory which show that by using suitable controls one dose of cod liver oil can be fed and effect a delay in onset of symptoms of vitamin A deficiency in proportion to the amount of vitamin A in that oil. We prefer to withhold further discussion of these results until the limitations of the method have been better established.

Our results on butter fat feeding are in agreement with those of Sherman (1), in so far as the growth response is concerned. Supplying vitamin D in generous quantities did not markedly affect the rate of growth during the test period. Vitamin D tests on the butter used showed that it had less than $\frac{1}{200}$ the vitamin D potency of a good grade of cod liver oil. The vitamin D requirement for growth of a rat at a rapid rate is apparently very low if the other constituents of the diet are supplied in proper proportions.

In comparing the responses we got in feeding butter fat with cod liver oil, it appears that the butter fat had about one-fifteenth the vitamin A potency of a good grade of cod liver oil. These results are in harmony with those of Jones, Steenbock, and Nelson (6). The exact history of the butter we used is not known. It was obtained during the month of April from the Bureau of Dairying and, although not as deep in color as butter obtained later in the year, could be considered a good grade of butter.

The authors wish to acknowledge the assistance of J. P. Devine and J. G. Wangler of this Division, in carrying out the experimental work described in this paper.

SUMMARY.

1. Data relating to the determination of vitamin A are presented, and certain defects of the United States Pharmacopœia vitamin A assay of cod liver oil are discussed.

2. The United States Pharmacopœia X method does not eliminate vitamin D as a growth-promoting factor. When this factor is provided ophthalmia usually develops before growth ceases, so that the curative test cannot be carried out on an animal free from disease.

3. In curative tests on animals which have developed incipient ophthalmia permanent cure of ophthalmia does not take place unless the level of vitamin A fed is high enough to permit growth of the rat at or near its maximum rate.

4. In curative tests for vitamin A, although observations on the presence and change of severity of ophthalmia are essential, the growth response seems to offer a more tangible means of judging vitamin A potency.

5. The vitamin A potency of a sample of butter fat was found to be approximately one-fifteenth of that of a good grade of cod liver oil.

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ON CEREBRONIC ACID. VI.

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In a recent paper Klenk[1] has emphatically refuted the accepted view of the structure of cerebronic acid as $C_{25}H_{50}O_3$. This statement seems very surprising in the light of the experience of these laboratories. Three different workers, each at a different time, have obtained lignoceric acid on oxidation of cerebronic acid. In the experiments reported by Levene and Taylor[2] the yield of lignoceric acid on oxidation of cerebronic acid was 81 per cent of the theory. A yield of this proportion could not be attributed to an impurity. Furthermore, the lignoceric acid obtained from the cerebronic acid was converted into the corresponding pentacosanic acid, which had the correct molecular weight, and the latter acid again was converted into a hydroxypentacosanic acid, which had the correct molecular weight. It is hard to conceive how Klenk considers his evidence more convincing than that furnished by Levene and West[3] and by Levene and Taylor.[2] However, in view of the very emphatic assertions of Klenk, we concluded to repeat the oxidation of cerebronic acid under more rigorous conditions than in the experiments of Levene and Taylor. Nevertheless, the main product of oxidation was an acid of the composition $C_{24}H_{48}O_2$, having the molecular weight of 369 (the theory requiring 368) and a melting point of 79.0–80.5°. Thus we shall adhere to the older view of the composition of cerebronic acid until Klenk shall have furnished more convincing evidence in favor of his theory. We may add, however, that we have some

[1] Klenk, E., *Z. physiol. Chem.*, 1928, clxxiv, 214.
[2] Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, 1922, lii, 227.
[3] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913–14, xvi, 475.

reason to believe that the acid $C_{23}H_{46}O_2$ of Klenk was a mixture of two acids, one of C_{24} and the other of C_{22}. A pure sample of the acid $C_{22}H_{44}O_2$ was obtained by us in an experiment in which a larger sample of cerebronic acid was oxidized by the usual method. The cerebronic acid in that experiment had a melting point of 99.5–100.5° and a specific rotation of $[\alpha]_D = +3.7°$.

EXPERIMENTAL.

Preparation of Phrenosin.—Mixed crude cerebrosides which had been kept in the laboratory for several years were purified in the following way. First, they were recrystallized several times from glacial acetic acid and this product was then recrystallized a few times from pyridine. The material was freed from pyridine by extraction with acetone. The dry residue was then recrystallized several times from a solution of equal parts (by volume) of methyl alcohol and of chloroform until further recrystallization no longer affected the specific rotation of the phrenosin. The rotation of this material in pyridine was $[\alpha]_D^{20} = +5.0°$.

Cerebronic Acid.—The cerebronic acid was obtained either by hydrolysis in an autoclave for 24 hours, or by alcoholysis by means of alcohol containing 8 per cent of sulfuric acid. The acid was purified by conversion into the lead salt and subsequent removal of the lead ion. Often the operation had to be repeated several times. The purified material melted at 130° and gave the following analysis:

4.355 mg. substance: 12.040 mg. CO_2 and 4.970 mg. H_2O.
0.500 gm. " required 12.55 cc. of 0.1 N NaOH.
$C_{22}H_{44}O_2$. Calculated. C 75.33, H 12.50, mol. wt. 398.
Found. " 75.39, " 12.76, " " 398.5.

The molecular weight was 398.5 and the optical rotation in pyridine was

$$[\alpha]_D^{25} = \frac{+0.14° \times 100}{1 \times 3.0} = +4.66°.$$

Oxidation of Cerebronic Acid.—Of the cerebronic acid just described, 24 gm. were oxidized in acetone solution with potassium permanganate, an excess of permanganate being employed. The oxidation was carried out in three lots, practically in the same

manner as described by Levene and Taylor, with the slight modification that each sample of 8.0 gm. of the acid was oxidized in 1000 cc. of acetone containing 10.0 gm. of potassium permanganate. By extracting the manganese dioxide with hot methyl alcohol the salts of the fatty acids were obtained, which yielded 14.0 gm. of fatty acids. These were fractionated in the manner described in the previous paper. Two fractions were obtained. The lithium salts were converted into the free acids, which were recrystallized from acetone. The larger fraction in this state of purity had the following composition.

4.140 mg. substance: 11.885 mg. CO_2 and 4.995 mg. H_2O.
$C_{24}H_{48}O_2$. Calculated. C 78.15, H 13.13.
Found. " 78.28, " 13.50.

The yield was 9.0 gm. It was then further purified by being passed through the lead salt. The acid then had the following composition.

3.775 mg. substance: 10.805 mg. CO_2 and 4.470 mg. H_2O.
0.3000 gm. " required 8.10 cc. of 0.1 N NaOH.
$C_{24}H_{48}O_2$. Calculated. C 78.15, H 13.13, mol. wt. 368.
Found. " 78.05, " 13.24, " " 370.

The molecular weight was 370. The acid melted (not absolutely sharply) at 79–80°. It was recrystallized several times from acetone and then from ether. The melting point was raised only slightly, 79.0–80.5°, and the molecular weight remained unchanged.

0.300 gm. substance required 8.13 cc. of 0.1 N NaOH. Mol. wt. 369.

The substance was then esterified. 5 gm. of the acid were taken up in 200 cc. of 98.5 per cent alcohol containing 2.5 gm. of sulfuric acid and refluxed overnight. On cooling, the ester settled out in plates. The ester was taken up in ether, washed free of adhering sulfuric acid, and the ester obtained on evaporation of the ether was recrystallized from alcohol. It melted sharply at 56–57°.

The mother liquor from the first crystallization was concentrated to 100 cc. and in the solution a deposit of glistening plates formed. The yield was 1.3 gm. It was dissolved in ether and freed from sulfuric acid. The residue from the ether was re-

crystallized from alcohol. The substance melted at 79–80°. It had the following composition.

0.0986 gm. substance: 0.2824 gm. CO_2 and 0.1156 gm. H_2O.
0.2000 " " required for neutralization 5.45 cc. of 0.1 N NaOH.
$C_{24}H_{48}O_2$. Calculated. C 78.15, H 13.13, mol. wt. 368.
Found. " 78.10, " 13.22, " " 367.

Hence this substance was lignoceric acid.

The ester melting at 56–57° was distilled. It distilled at 205–210°, leaving only a small residue. The distillate melted at 56–57°. The ester was converted into the free acid in the usual way. The acid, after recrystallization from benzene, melted at 78–79° and solidified at 76°. It had the following composition.

0.0986 gm. substance: 0.2830 gm. CO_2 and 0.1156 gm. H_2O.
0.2000 " " required for neutralization 5.43 cc. 0.1 N NaOH.
$C_{24}H_{48}O_2$. Calculated. C 78.15, H 13.13, mol. wt. 368.
Found. " 78.26, " 13.22, " " 368.3.

The lower melting points may be due to the fact that the cerebronic acid was obtained from cerebrosides prepared many years ago, or perhaps to slight admixtures of the lower acids to be described in another paper.

SYNTHESIS OF VITAMIN B IN THE RUMEN OF THE COW.*

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(Received for publication, September 14, 1928.)

In our studies concerning the vitamin B requirement of calves, one of us produced conclusive evidence that a calf will grow normally to maturity and produce normal offspring on a ration that carries an insufficient amount of the vitamin B complex to support growth and well being in rats (1). In later investigations we determined that vitamin B in milk is not dependent on the presence of this vitamin in the ration of the cow (2). Three cows that were fed for over 2 years, throughout their growth period, on a ration that was decidedly deficient in vitamin B, were used in this study. The evidence obtained in these investigations appeared to indicate that cattle, and possibly all other ruminants, possess the ability to synthesize vitamin B. Damon (3), Kuroya and Hosoya (4), Heller *et al.* (5), Scheunert and Schieblich (6), Sunderlin and Werkman (7), and others have made contributions to our knowledge of the possible synthesis of vitamin B complex by bacteria. The present paper is intended to set forth the results of an investigation which was designed to determine whether the microorganisms present in the rumen of one of our experimental cows were responsible for the synthesis of vitamin B complex.

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EXPERIMENTAL.

A pure bred Holstein heifer, Penstate Homestead Jessie, No. 924062, was made the subject of this study. She was one of a group of seventeen experimental animals, and had grown to maturity on the experimental ration, which was made up from corn gluten meal, cane sugar, commercial casein, polished rice, corn-starch, pearled hominy, dried sugar beet pulp, cod liver oil, and mineral supplements. This ration was demonstrated, through rat feeding trials (1), to be practically devoid of the vitamin B complex.

A permanent fistula about 3½ inches in diameter was made in the rumen of the experimental heifer through the left side. This provision afforded an easy means of sampling the rumen contents. A special pack, after the method used by Schalk and Amidon (8), was provided for keeping the fistula tightly closed so that normal conditions within the rumen would be maintained. The heifer continued to thrive (on the ration deficient in vitamin B) and gave every evidence of functioning normally as she had done on the experimental ration, prior to the operation.

Our first step in the investigation consisted in testing out alcoholic extracts from the fermented rumen contents. 12 hours after feeding, portions of about 30 pounds of the wet fermented feed were removed from the rumen and placed in a 5 gallon milk can. The fermented material was incubated for 5 days at a temperature of 37°, with occasional stirring of the mixture in order that the bacterial flora might multiply and produce, if possible, the maximum quantity of vitamin B. This procedure was considered preliminary, and precautions were taken to provide an extract sufficiently concentrated to eliminate possible difficulties in feeding technique. A total of 56,530 gm. of the fermented rumen contents, representing four separate samplings, was used in preparing the extract. This represented 6252 gm. of dry matter, the average percentage of dry matter being 11.06 per cent.

The fermented rumen contents were stored in 5 gallon stone jars. 95 per cent ethyl alcohol was added to the jars in liberal amounts because of the large amount of moisture present. It was the aim to provide a solution of approximately 70 per cent alcohol. The jars containing the mixture were allowed to stand for 1 week at room temperature with occasional stirring each day.

The liquid was then filtered off and evaporated to dryness at a temperature of 50°, after the usual practice of preparing vitamin B extracts. 2226 gm. of extract carrying 1200 gm. of dextrin, were so prepared that 1 gm. of this extract represented 25.4 gm. of the original fresh fermented rumen material.

Rat Feeding Experiments with Rumen Extract.

The feeding technique used in this and later feeding trials is essentially that described by Dutcher, Francis, and Combs (9). The animals were placed on experiment when they were 21 days of age, and their average weight was about 40 gm. Males and females were used in equal numbers, and litter mates were distributed throughout the groups. Each rat was kept in an individual cage provided with a screen bottom (10). Growth records were obtained and food intake noted.

The animals refused to eat the ration containing rumen extract for the reason that it carried a decided butyric acid odor. 3 per cent of olive oil incorporated in the ration proved to make it palatable. The ration finally adopted (Ration 103) was made up as follows:

	per cent
Casein	18
Salts 185*	3
Agar	2
Olive oil	3
Dextrin	24
" rumen extract	50
	100

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxi, 181.

The controls also receive an equivalent of olive oil in their ration. Each rat was given 4 drops of Squibb's cod liver oil daily to insure an adequate supply of vitamins A and D.

The average growth curve for the two groups is shown in Chart I. In the 8 week period the groups receiving the rumen extract made an average gain in weight of 5.2 gm. per week (Curve I).

The growth curves indicate clearly that the rumen extract must have supplied the vitamin B complex since all animals maintained a constant rate of growth throughout the 8 week period. The

controls (Curve II, Chart I) were significantly different although they continued to live (some of them for as long as 10 weeks), but their increases were very slight during the 8 week period.

The evidence obtained in this feeding experiment seemed to indicate quite clearly that vitamin B must have been synthesized by bacteria or other microorganisms. The next step was to make a study of the microflora in the rumen.

Bacterial Study of Rumen Microflora.

The work of Damon (3) on acid-fast bacteria which synthesized the vitamin B complex, suggested that we might be dealing with a similar situation in the rumen of our experimental cow. We had also expected to find wild yeasts. Direct microscopic examination

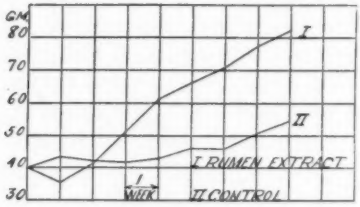

CHART I.

of stained smears of the rumen contents revealed the presence of neither acid-fast bacteria nor yeasts. Samples plated with plain nutrient agar revealed approximately 2,225,000 bacteria per gm. of rumen contents but no yeast colonies were found. What we did find was an almost pure culture of a bacterium that produced a small colony about 1 to 2 mm. in diameter. The colonies developing upon the surface showed a pale lemon-yellow pigment. The subsurface colonies developed no pigment but when transfers were made to agar slants, they proved to be identical with the surface colonies. A search of the literature revealed that our organism had never been described. We have, therefore, designated it *Flavobacterium vitarumen*. Platings on plain nutrient agar of several samples showed the results indicated in Table I. The predominance of this type of colony in our first plates prompted us to make preparations for a test of its ability to synthesize the vitamin B complex.

Plain nutrient agar medium, made from the dehydrated Bacto-Nutrient Agar manufactured by the Digestive Ferments Company, was placed in 32 ounce bottles of the Blake type and sterilized for 20 minutes at 15 pounds pressure. The bottles were placed in a horizontal position in order that the agar would solidify on one side of the bottle and provide the maximum area for growth. The media in these bottles were then inoculated with a water suspension of a pure culture of the organism and incubated for 48 to 72 hours at 37°. The growth, which was not abundant, was then washed from the surface of the media with sterile water and the washings were evaporated to dryness at a temperature of approximately 50°. The amount of growth secured in this manner was less than 1 gm. of dried material per bottle. We were

TABLE I.

Date.	Total No. of bacteria per gm.	Approximate percentage of *Flavobacterium vitarumen*.
Feb. 21	2,225,000	98
" 26	8,500,000	98
Mar. 4	1,840,000	97
" 17	1,850,000	96
June 30	26,300,000	95
July 13	27,450,000	90
Aug. 1	34,500,000	50

able, however, to prepare a sufficient quantity of dried bacterial cells in the form of a meal to make the necessary test for vitamin B in the rat feeding experiments.

Flavobacterium vitarumen is rod-shaped, being 0.5 to 1.5 microns × 0.5 — 3.0 microns in size. It has rounded ends, does not form endospores, generally occurs singly but occasionally in pairs, and is non-motile. It differs from the vast majority of the genus *Flavobacterium* in that it is Gram-positive.

Surface agar colonies are 1 to 4 mm. in diameter, while the imbedded colonies are somewhat smaller. Gelatin colonies are very similar to the agar colonies. Other cultural characteristics are as follows: Agar slant, filiform; chromogenesis, pale lemon-yellow; gelatin stab, no liquefaction; sugar broths, no gas produced; sucrose, dextrose, and maltose broth, acid produced;

lactose, xylose, dulcitol, mannitol, sorbitol, and insitol broth, no acid produced; starch agar, no diastase; cellulose, no fermentation; lead acetate agar, no growth; Endo's medium, no growth.

Litmus milk is fermented with the production of acid. There is a slight reduction of the litmus but no curd is formed.

It does not form a ring of pellicle growth on any of the broth media. The growth clouds the liquid and finally settles. It does not form indole or skatole and nitrates are reduced.

Rat Feeding Experiments with Dried Bacteria.

The feeding technique was the same as that employed in the previous test on the rumen extract. The dried bacterial meal

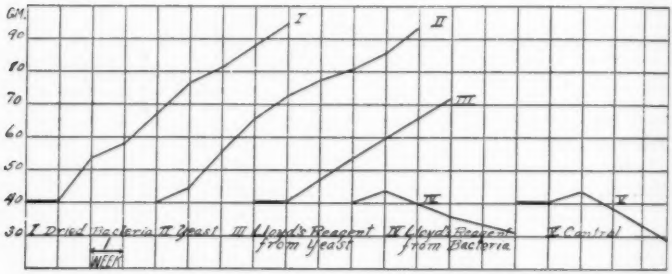

CHART II.

was fed daily in 0.5 gm. doses to each rat as a supplement to the basal ration. In Curve I, Chart II, the result of this feeding test is presented. The curve represents the average growth of ten rats. The average weekly gain per rat was 6.8 gm.

At the same time another group of rats, some of which were litter mates of those represented in Curve I, were fed the basal ration supplemented with 0.5 gm. of Fleischmann's special yeast. In Curve II the average growth of this group is presented. The average weekly gain per rat in this group was 6.4 gm.

There was some question as to whether the media (dehydrated nutrient agar) which were used in growing the large quantities of bacterial cells, did not carry appreciable amounts of the vitamin B complex. To test out this point, a liter of medium made from Bacto-Nutrient Agar was shaken with 10 gm. of Lloyd's reagent.

The mixture was filtered and the filtrate evaporated to dryness. The residue was fed to rats in daily 0.5 gm. doses with the result as presented in Curve IV, Chart II. A similar test was made with autolyzed yeast, prepared as described by Seidell (11). Curve III represents the average growth of this group. A control group was also fed on the basal diet with the results portrayed in Curve V.

SUMMARY AND CONCLUSION.

Investigations were conducted on the fermented rumen contents of a Holstein cow, representative of a group of seventeen animals that were grown to maturity on a ration highly deficient in vitamin B complex. Alcoholic extracts of the fermented rumen contents were proved potent in the vitamin B complex through rat feeding trials.

One bacterium of the genus *Flavobacterium* was found about 90 per cent predominant in the rumen microflora. This organism was grown in large quantities on vitamin B-free media and fed to rats to the extent of about (12 per cent) of dried bacterial cells in a synthetic vitamin B-free ration. This test proved the bacterial cells to be highly potent in the vitamin B complex.

The results of this study warrant the conclusion that the vitamin B complex was produced in the rumen of our experimental cow by bacterial fermentation. This result offers a satisfactory explanation as to why cattle, unlike any other species of animal yet studied, have the ability to grow to maturity, to produce normal offspring, and to produce milk of normal dietary composition, on a ration that carries an insufficient amount of vitamin B complex to support growth and well being in rats.

Credit is due Dr. J. F. Shigley, of the Department of Animal Husbandry, for veterinary service in caring for the experimental heifer with the permanent fistula.

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XUM

STUDIES ON THE COMPOSITION OF HUMAN MILK.*

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Numerous reports concerning the composition of human milk and colostrum are to be found in the literature, but most of them are based on only a few cases. The present investigation includes analyses of the milk of 88 normal women for protein, sugar, fat, and in 60 of these cases for ash. Two samples were obtained from each subject, one on the 5th and one on the 9th day post partum, and one or two additional samples were procured from 50 of these subjects at later periods, ranging from 3 to 8 weeks post partum. The average composition of the milk at different periods and the variations observed were studied and compared with the results of others. An attempt was also made to investigate the effects, if any, of different diets on the composition of the early milk. Ordinary hospital diet, high carbohydrate, and high fat diets were used. The infants of the above mothers and others who were fed the same diets but whose milk was not analyzed—130 in all—were weighed daily while in the hospital. 75 of these were brought back to clinic one or more times and were weighed at each visit. An average growth curve has been plotted from these weights.

Procedure and Methods.

The diets used were as follows: Diet 1, ordinary hospital diet; Diet 2, ordinary hospital diet plus 60 gm. of rice daily; Diet 3, ordinary hospital diet plus 60 gm. of tapioca daily; Diet 4, ordinary hospital diet plus 100 gm. of 40 per cent cream daily. Diets 2, 3, and 4 were calculated to have the same caloric value; namely,

* Preliminary reports of this work have been published by Kleiner and Bell (7) and Kleiner, Tritsch, and Graves (8). The latter article stresses the clinical side of the question.

about 3000 calories. Diet 1 contained about 2600 calories. Each mother was given the specified diet from the time of delivery until she left the hospital. She was encouraged to continue the same diet at home, but since this condition could not be supervised no conclusions have been drawn regarding the effect of diet after the patients left the hospital.

The milk was obtained in every case by an electric breast pump, one breast—the one not used at the previous nursing—being completely drained. On the 5th and 9th days post partum the milk was taken at approximately 9 a.m. Later samples after the patients left the hospital were taken in the afternoons when the mothers returned to the hospital clinic. The babies were weighed at these times also.

In nearly all cases the analysis of the milk was started immediately after the samples were obtained. Occasionally specimens were placed on ice until the next day, but in no case was the analysis postponed more than 24 hours. The complete sample was mixed and the volume and specific gravity were taken. Fat was then determined by the Babcock method, protein by the Kjeldahl method (total nitrogen $\times$ 6.37), lactose by the Owen and Gregg (10) modification of the Folin and Wu blood sugar method, and ash by evaporation with concentrated nitric acid and ignition at a low temperature.

Results.

The averages of all samples of milk analyzed are shown in Table I. Since wide variations occurred among the different cases maxima and minima for each period are included. These averages and the weight curves of the infants are plotted in Chart I. An example of the variations in the milk at different periods is given in Table II, which is a record of a typical case on ordinary hospital diet.

In considering the averages of all analyses, the following observations have been made. The protein content of the milk decreased rapidly at first, then more slowly. The fat and sugar increased, first rapidly, then slowly, until a constant level was reached and maintained. Ash decreased from the 5th to the 9th day, but was not studied further. These average tendencies correspond with those observed by Hammett (5) but are not

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always exhibited in individual cases. Table III compares the present results with those of Hammett and other workers. In Table IV are shown the individual variations in the change of composition from the 5th to the 9th day. In a small number of instances the protein and ash increased from the 5th to the 9th day, and even more frequently the lactose and fat showed decreases during that period. All components of the milk were quite variable, but the fat fluctuated the most, both in the same individ-

TABLE I.

Average Composition of Human Milk at Different Periods.

Time.	No. of cases.	Protein.			Sugar.			Fat.		
		Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
5 days.	88	1.45	2.83	2.00	4.62	7.37	6.42	0.9	8.2	3.2
9 "	88	1.12	2.65	1.73	4.76	7.65	6.73	1.6	7.1	3.7
3–4 wks.	35	1.03	1.79	1.37	6.17	7.89	7.11	1.4	6.1	3.6
5–6 "	32	0.98	1.57	1.30	5.97	8.33	7.11	1.3	7.6	4.0
7–8 "	14	1.04	1.40	1.21	6.25	7.83	7.11	1.1	7.0	4.0

TABLE II.

Composition of Milk of One Woman at Different Periods.

Days post partum.	Volume.	Sp.gr.	Ash.	Protein.	Sugar.	Fat.
	cc.		*per cent*	*per cent*	*per cent*	*per cent*
5	60	1.037	0.28	2.20	6.51	0.9
9	113	1.032	0.27	2.00	6.86	5.6
28	33	1.032		1.48	7.05	3.3
42	32	1.032		1.32	6.95	4.4

ual at different periods and in different individuals at corresponding periods. Hammett's individual cases, however, also fail to accord at all times with the group averages.

The present investigation agrees with Hammett's findings that the range of variation of the protein and lactose narrows in later periods, but the fat remains eratic (Table II). The volume of milk obtained by completely emptying one breast was also

extremely variable. On the other hand, the results here reported do not bear out Hammett's observation of a uniform production plane for the individual. Of the 88 cases studied, 58, or 66 per cent, had a protein level consistently above or below the group averages, but only 46, or 52 per cent, maintained a uniform lactose level, and only 36, or 41 per cent, a uniform fat level.

In comparing his results with those of European (1, 2, 4, 12)

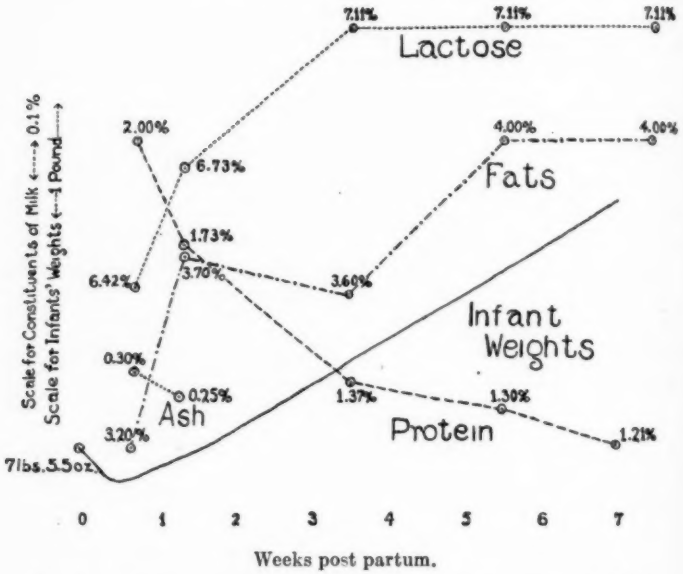

CHART I. Average composition of human milk at different periods, compared with the average weight curve of the infants. The different constituents are plotted on different base lines but the scale is the same in all cases.

and other American (9) investigators, Hammett concludes that the milk of American mothers is higher in lactose and lower in protein than that of European women. The European figures for lactose vary from 3.86 per cent to 5.99 per cent in milk of the 5th to the 11th days post partum, while the American figures quoted are 6.17 and 6.53 per cent. The average for the 5th and 9th days of the 88 cases here discussed is 6.57, agreeing with the other American reports. However, the present results for protein during the

TABLE III.

Comparison of Averages of Milk Analyses at Different Times after Parturition, Reported by Various Authorities.

Investigator.	Days post partum.	Ash.	Protein.	Sugar.	Fat.	No. of cases.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*		
Gardner and Fox (3)...	1–6	0.271	2.39	7.03	3.08	179	Average of compilation of results of many other investigators.
Hammett (5).....	3		3.52	5.43	4.34	8	
Holt, Courtney, and Fales (6).....	3–5	0.33	1.96	6.50	3.30	6	Analysis of composite sample.
Hammett.....	5		1.74	6.08	2.88	8	Same cases as on 3rd day.
Present.....	5	0.30	2.00	6.42	3.20	88	
von Gorup-Besanez (4).							
Clemm.....	5–11			3.86			
Tidy.....	5–11			5.14			
Camerer and Söldner (1, 2), Söldner (12)..	5–11		1.77	5.99		13	Composite samples.
Meigs and Marsh (9)..	5–11		1.55	6.53		5	" "
Hammett.....	7		1.61	6.21	3.80	8	Same cases as on 3rd day.
Gardner and Fox.....	7–12	0.237	1.89	6.79	3.42	100	Average of compiled figures.
Holt, Courtney, and Fales.....	5–12	0.31	2.19		3.05	4	Composite sample.
Hammett.....	9		1.69	6.32	3.70	8	Same cases as on 3rd day.
Present.....	9	0.25	1.73	6.73	3.70	88	Same cases as on 5th day.
Schlossmann (11).....	9–10		1.85	6.92	4.23	6	
Hammett.....	11		1.46	6.42	3.38	8	Same cases as on 3rd day.
Schlossmann.....	11–20		1.84	6.89	4.63	25	
Holt, Courtney, and Fales.....	12–30	0.241	1.56		4.37	6	Average of 6 analyses, individual and composite.

TABLE III—*Concluded.*

Investigator.	Days post partum.	Ash.	Protein.	Sugar.	Fat.	No. of cases.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*		
Gardner and Fox.....	13–30	0.216	1.73	6.96	3.94	220	Average of compiled figures.
Present.....	18–31		1.37	7.11	3.60	35	
Schlossmann.....	21–30		1.97	6.77	4.53	41	
"	31–40		1.78	6.80	5.41	21	
Present.....	32–45		1.30	7.11	4.00	32	
Schlossmann.....	42–49		1.78	6.80	5.41	13	
Present.....	46–61		1.21	7.11	4.00	14	
Schlossmann.....	51–60		1.59	7.28	4.62	24	

same period average 1.86 per cent, agreeing better with the 1.77 per cent of the European workers than with the 1.55 and 1.62 per cent of the American figures.

When the average weights of the infants were studied it was found that a minimum, averaging 8.4 ounces below birth weight, was reached on the 3rd day. Thereafter a steady increase in weight occurred until an average of 0.4 ounce below birth weight was reached on the 11th day, at which time most cases left the hospital. Of 130 cases studied, 56, or 43 per cent, had regained or passed their birth weight on leaving the hospital, about the 11th day post partum, and 74, or 57 per cent, were still below. The average gain in weight after leaving the hospital was about 9 ounces a week. A careful analysis of the data failed to show any relationship between the composition of the early milk and the gain or loss in weight of the infant.

The different diets fed to the mothers in this investigation had very little observed effect on the composition of the milk (Table V). All four diets were theoretically adequate, the differences being in the addition of an excess of carbohydrate in Groups II and III and an excess of fat in Group IV. The variations in composition of the milk of individuals on any one diet were so much greater than the differences in the respective group averages that it is difficult to draw conclusions.

The average volume of milk was greatest in the high carbohydrate and least in the high fat group. The milk of the group fed on ordinary hospital diet was higher in protein on both the 5th and the 9th days than that of any of the other groups. This diet contained the same quantity but a higher percentage of protein than the others. The milk sugar was higher in both the high carbohydrate and high fat groups on the 5th day than in the control group, but the sugar was about the same on the 9th day in all groups. The high fat diet apparently increased the fat content of the milk on the 5th day but had no effect on the 9th. It would seem, therefore, that feeding of high carbohydrate or fat may raise the milk sugar, or of fat may raise the milk fat, to a slightly higher

TABLE IV.

Changes in Composition of Milk of Individual Women from 5th to 9th Day Post Partum.

Group No.	Protein. No. of cases.		Lactose. No. of cases.		Ash. No. of cases.			Fat. No. of cases.		
	Decreased.	Increased.	Decreased.	Increased.	Decreased.	Same.	Increased.	Decreased.	Same.	Increased.
I (control)	15	2	2	15	16	0	0	6	1	10
II (rice)	19	1	4	16	17	1	0	6	0	14
III (tapioca)	25	5	8	23	15	1	2	7	1	23
IV (cream)	17	2	6	14	3	0	0	11	0	9
All	76	10	20	68	51	2	2	30	2	56

level during the period when they tend to be low, but as they naturally increase will not raise them above their normal maxima.

No correlation between the age or parity of the mother and the composition of the milk could be found.

SUMMARY.

1. Reports of analyses of breast milk of 88 normal women are presented.

2. The average figures for ash and protein decreased and for lactose and fat increased with the time interval post partum. Individual cases frequently failed to follow this trend.

TABLE V.

Composition of Early Milk of Women on Different Diets.

Group No.	Days post partum.	No. of cases.	Volume.			Sp.gr.			Ash.			Protein.			Sugar.			Fat.		
			Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.
			cc.	*cc.*	*cc.*				*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I. (control).	5	17	10	86	59	1.031	1.037	1.034	0.28	0.34	0.30	1.75	2.74	2.13	4.62	7.18	6.10	0.9	4.2	3.1
	9	17	14	113	72	1.028	1.037	1.033	0.20	0.33	0.25	1.59	2.65	1.82	5.37	7.55	6.70	1.7	6.4	3.8
Average					65.5			1.0335			0.275			1.975			6.40			3.45
II. (rice).	5	20	13	190	72	1.027	1.036	1.034	0.24	0.36	0.29	1.50	2.54	1.99	4.75	7.07	6.28	1.6	7.5	3.25
	9	20	11	149	68	1.028	1.036	1.033	0.21	0.27	0.24	1.33	2.08	1.69	5.86	7.53	6.85	1.9	7.1	3.8
Average					70			1.0335			0.265			1.84			6.565			3.5
III. (tapioca).	5	31	6	348	72	1.030	1.037	1.033	0.25	0.36	0.30	1.60	2.83	1.99	5.29	7.37	6.58	0.9	5.0	3.0
	9	31	10	386	67	1.029	1.036	1.033	0.18	0.35	0.26	1.12	2.17	1.72	4.76	7.48	6.64	1.9	6.1	3.6
Average					69.5			1.033			0.28			1.855			6.61			3.3
IV. (cream).	5	20	13	119	60	1.027	1.037	1.033				1.45	2.28	1.94	5.79	7.33	6.59	1.4	8.2	3.6
	9	20	6	112	54	1.027	1.037	1.033				1.42	2.21	1.73	5.40	7.65	6.77	1.6	6.2	3.8
Average					57			1.033						1.835			6.68			3.7

3. A comparison with the results of other investigators has been made.

4. Supplementary feeding of carbohydrate and of fat made only slight differences in the composition of the early milk. The average figures for milk sugar were a little higher in both of the above groups than in the controls and the milk fat was highest in the group receiving supplementary feeding of fat. The average volumes secreted, however, were greater after supplementary feedings of carbohydrate and less after fat feeding than in the controls.

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THE AVAILABILITY OF ERGOTHIONEINE IN SUPPLEMENTING RATIONS DEFICIENT IN HISTIDINE.

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INTRODUCTION.

Cox and Rose (1926, *b*) have fed imidazole derivatives to young rats restricted to diets deficient in histidine. Their purpose was to test whether any preformed imidazole derivative could serve in place of the essential amino acid histidine in the ration. Of the series studied by them, only one, β-4-imidazole lactic acid, enabled the rats to grow. They also attempted to substitute purines, creatinine, and creatine for histidine but found no replacement value in these substances (Cox and Rose, 1926, *a*). Harrow and Sherwin (1926) have confirmed the findings of Cox and Rose in regard to imidazole lactic acid and have also found that 4-imidazole pyruvic acid can serve in place of histidine, though not as efficiently as the lactic acid derivative. They also claim some replacement by β-4-imidazole acrylic acid (urocanic acid) as opposed to the entirely negative findings of Cox and Rose.

Of the compounds used in the above studies, urocanic acid is the only one associated with animal metabolism that has a structure closely related to histidine. Other known imidazole derivatives identified in animal tissues might possibly support growth if added to a diet deficient in histidine. Growth may result either by sparing dietary histidine that is required for the synthesis of the imidazole derivative in question or by conversion to histidine. Ergothioneine, $C_9H_{15}N_3O_2S$ (Tanret, 1909), the betaine of 2-thiolhistidine (Barger and Ewins, 1911), recently discovered as a normal component of animal blood (Hunter and Eagles, 1925,

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1927; Benedict, 1925; Benedict, Newton, and Behre, 1926; Eagles and Johnson, 1927; Newton, Benedict, and Dakin, 1927) is such a substance. We have carried out a study of its histidine value in the diet.

EXPERIMENTAL.

A healthy litter of ten young albino rats was fed a ration similar in composition to the histidine-deficient diet of Cox and Rose. The histidine-deficient amino acid mixture of Diet D (see Table I) was the same as that previously employed, excepting that silver

TABLE I.

Diet D (Histidine-Deficient Diet).

	gm.
Completely hydrolyzed casein minus histidine	14.5
Cystine	0.3
Tryptophane	0.2
Dextrin	40.0
Sucrose	15.0
Lard	19.0
Cod liver oil	5.0
Salt mixture (Osborne and Mendel).*	4.0
Agar	2.0
Total	100.0

Diet E was prepared by adding 0.545 gm. of ergothioneine to 100 gm. of Diet D. Diet H was prepared by adding 0.5 gm. of histidine monohydrochloride to 100 gm. of Diet D.

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

oxide (Vickery and Leavenworth, 1927) was used to precipitate the histidine. Arginine was not precipitated. A preparation containing sufficient histidine to give a maintenance ration or only slight growth was selected. On such a ration a rat should show an increased growth rate if any added substance has even the slightest value in replacing histidine. The basis of this selection from several preparations was an approximately quantitative determination of the histidine content of the amino acid mixture by means of the intensity of color developed in Hunter's (1922) modification of Knoop's test. Vitamin B was supplied by 200 mg. of dried brewers' yeast daily, instead of the Harris Vitamine

Powder previously used by Cox and Rose. The yeast certainly contributed some histidine to the diet and consequently more rapid growth was obtained on the deficient diets than was expected.

The ergothioneine used was from the preparations of Hunter and Eagles (1925, 1927). The authors gratefully acknowledge their indebtedness to Professor George Hunter of the University of Toronto for the supply of ergothioneine.

The method of caring for the rats was the same as that of Cox and Rose excepting that no preliminary ration was employed.

TABLE II.

Food Consumption and Body Weight Changes.

Days	1–4			5–12			13–32			33–52			53–72		
Average daily:	Growth.	Food.	Diet.	Growth.	Food.	Diet.	Growth.	Food.	Diet.	Growth.	Food.	Diet.	Growth.	Food.	Diet.
	gm.	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*	
Rat 949 ♀	−1.5	4.5	H	+0.4	4.5	H	+2.3	5.9	H	+1.5	8.1	H			
" 950 ♂	−2.0	5.1	"	+0.8	5.8	"	+1.4	4.5	"	+0.9	6.2	D			
" 951 ♀	−0.8	5.1	"	+0.5	6.0	"	+2.2	5.6	"	+0.3	5.7	E	+1.9*	7.7	H
" 952 ♀	−1.8	4.8	"	+0.8	6.6	"	+1.5	5.0	"	+0.8	6.4	D			
" 953 ♀	−2.5	4.0	D	−0.6	3.8	D	+0.7	4.0	D	+0.8	5.0	E	+0.5†	3.7	D
" 954 ♀	−2.8	3.5	"	−0.5	5.3	"	+0.6	4.2	"	+2.1	7.0	H	−0.4	5.8	"
" 955 ♂	−2.3	4.5	H	+0.1	4.8	H	+1.5	4.6	H	+0.6	5.8	E	+0.6	5.5	"
" 956 ♂	−1.3	6.0	"	+0.5	5.5	"	+1.1	4.8	"	+1.5	6.5	H			
" 957 ♂	−1.5	6.0	"	+0.6	5.0	"	+2.0	5.4	"	+1.2	6.6	"			
" 958 ♀	−1.5	5.5	"	+0.6	5.5	"	+1.8	5.8	"	+0.3	6.8	D	+2.0‡	7.4	H

* Only 53 to 68 days.

† An additional period, 73 to 84 days, showed an average daily gain of +2.7 gm. and an average food consumption of 5.3 gm. on Diet H.

‡ Only 53 to 68 days.

Eight of the rats were placed on the deficient diet plus 0.5 per cent of histidine monohydrochloride (Diet H); two, Rats 953 and 954, were started on diets with no added histidine (Diet D). After a period of adjustment, satisfactory growth began in all cases. The two rats on the deficient diet lost weight for a longer time than those receiving histidine and then began to grow at a much slower rate. After 32 days the 0.5 per cent histidine monohydrochloride was removed from the ration of some of the rats, and in the cases of

two of them an equivalent amount (0.545 per cent) of ergothioneine was added. Ergothioneine and histidine were included at this time in the diets of Rats 953 and 954 respectively. The

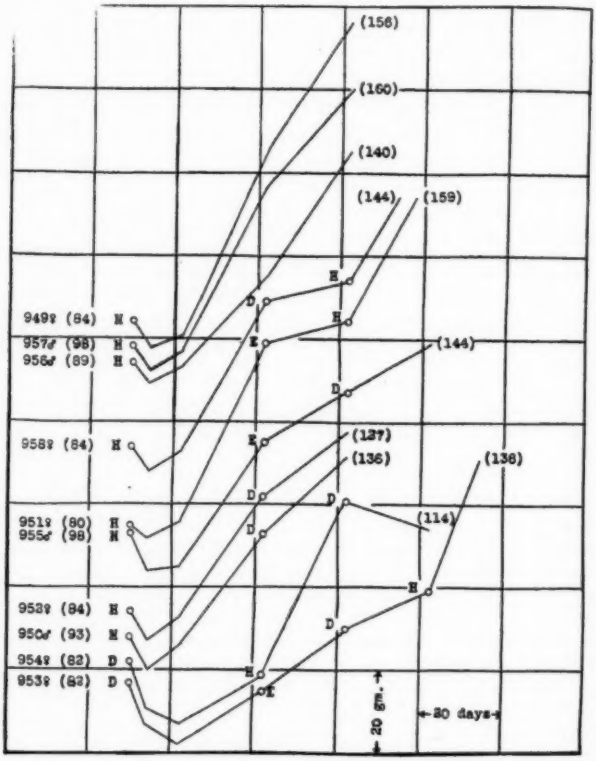

CHART I. The numbers in parentheses at the beginning and end of each growth curve of Chart I are the initial and final weights in gm., respectively, of each rat. Change of ration is indicated on each curve by a small circle. The letters D, E, and H placed near the small circles show that Diets D, E, or H, respectively, were employed in the succeeding period.

experimental period during which the ergothioneine was fed was 20 days. At the end of that time the rats were continued on either the adequate or the deficient diets.

The data of growth and food consumption are presented in Table II, and the growth is shown graphically on Chart I.

DISCUSSION.

The data on the average daily consumption of food, which was in all cases offered *ad libitum*, show in general a greater intake of the diet supplemented with histidine. Complete adjustment of all the rats to the rations employed occurred about the 12th day of the experiments. Thereafter the following facts are apparent from Table II.

Growth was always more rapid on the ration supplemented with histidine. When that amino acid was included in the diet the *minimum* growth rate obtained was greater than the *maximum* rate of any rat on either the deficient diet or the ration supplemented with ergothioneine (see Rat 956, period 13 to 32 days, and Rat 950, period 33 to 52 days).

When the rations of the rats were changed, significant alterations in the growth of the animals occurred. Changes of diet from the deficient, or the deficient plus ergothioneine, ration to that supplemented with histidine resulted in the greatest growth rates (Rats 951, 953, 954, and 958). The reverse change from an adequate to the deficient or ergothioneine ration always resulted in retarded growth (Rats 950, 952, and 958; Rats 951 and 955). Changes of diet from the deficient to the deficient plus ergothioneine ration or *vice versa* caused no significant change in the growth rate (Rats 953 and 954).

CONCLUSIONS.

No evidence has been found to indicate that ergothioneine can be converted to histidine by the young rat or can diminish the histidine requirement of the growing animal.

The authors acknowledge the suggestion of Dr. R. W. Jackson of Yale University that they undertake this investigation jointly.

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TOTAL SUGAR OF BLOOD AND URINE.

II. THE HYDROLYZABLE SUGAR OF BLOOD.

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In a recent paper (1) a method for the estimation of total sugar of blood and urine was described, and values for the hydrolyzable sugar of deproteinized filtrate of whole blood were given. We are now reporting similar values for plasma filtrate.

The separation of corpuscles and plasma of the venous blood of normal adults was made by the technique of Folin and Berglund (2). The non-protein plasma filtrates were prepared by the method of Folin and Wu (3). Clotting occasionally occurred in the plasma of blood drawn from the external jugular vein of animals. In such cases plasma serum was used for the preparation of the protein-free filtrate. In the future it is planned to take mammalian blood from the veins of the extremities, and to use 5 cc. total sugar tubes for the hydrolysis, and 0.5 cc. Ostwald-Folin pipettes for the addition of the acid and alkali, to reduce the volume of blood required.

The free and hydrolyzable sugar were determined by the colorimetric methods of Folin and Wu (4), Benedict (5, 6), Folin (7), an adaptation of Kingsbury's method (8), and a modification of Sumner's method (1). The Folin-Wu values were corrected for proportionality in two ways. The corrections of Oser and Karr (9) were used for all values exceeding 50 mg. per cent, and the corrections described on p. 260 for values from 4.7 to 25 mg. per cent.

Further evidence of the correctness of the original hydrolytic procedure (1) has been obtained. Increasing the concentration of the sulfuric acid solution to 9.0 N led to decreased values for the hydrolyzable sugar. Lengthening the period of hydrolysis from 1¼ to 5 hours proved to be of no practical advantage in the analysis

of normal blood. That hydrolysis does not increase the amount of reducing nitrogenous substances in blood filtrate has been demonstrated by means of the ammoniacal copper reagent of Folin and Svedberg (10), which is reduced by creatinine, uric acid, *etc.*, but not by glucose. Several blood filtrates, after hydrolysis by the total sugar method, gave slightly lower reductions than the original filtrates.

Benedict (6) has suggested that substances dissolved from filter paper increase the apparent amount of sugar. Whatman paper used in our experiments was macerated for 2 hours with amounts of distilled water equivalent to the volume of blood filtrate prepared, and the extract carefully analyzed. The amounts of free and hydrolyzable sugar extracted were so small as to be entirely within the limits of experimental error. Similar analyses of extracts of the oxalate cloth in routine use in this laboratory were made after shaking the cloth with the appropriate amount of 0.01 per cent glucose standard for 3 minutes. The effect of the cloth was found to be negligible.

In addition to silicates, ferric iron has an inhibitory action on copper reduction, but the iron must attain a concentration of 0.005 per cent in the glucose standard before it has a measurable effect. Hence, neither potassium hydroxide nor blood filtrates contain sufficient ferric iron to affect the analytical values.

We have assumed, heretofore, that mixtures of sugars are merely additive in their reducing action. This assumption has been verified by analyzing mixtures of sugars having widely divergent equivalents, such as maltose and glucose, and maltose and xylose.

The experiments recorded in Table I show that the major portion of the hydrolyzable sugar of blood is derived from the formed elements, but the plasma also contains a small amount. After a night's fast (Samples 31, 37, 38), the hydrolyzable sugar was still present in both corpuscles and plasma. Folin and Berglund (2) reported negative values for the hydrolyzable sugar of plasma, as determined by the Folin-Wu method. It has been previously pointed out that their results were partly due to the salt effect of the sodium chloride, and partly to the presence of silicates (1).

When blood contains as much free sugar as Sample 25, the possibilities of error in the hydrolyzable sugar estimation are greatly increased, explaining the negative values for this sample

TABLE I.

Distribution Experiments.

Sample No.	Normal human.	Mg. per cent of sugar.							
		Free.				Hydrolyzable.			
		Folin-Wu.	Benedict.	Folin.	Sumner.	Folin-Wu.	Benedict.	Folin.	Sumner.
7	Whole blood.	98	91			12.5	17		
	Plasma.	101	94.5			3.5	−1		
8	Whole blood.	98		83		12.5		13	
	Plasma.	99.5		115		2		−30.5	
9	Whole blood.	82.5	77	71		15	9	10	
	Plasma.	90	78	78		1.5	1.5	− 5	
10	Whole blood.	98	91	83		16	17	13	
	Plasma.	104	96	100		5	12	−15	
13	Whole blood.	73.5	70.5			14	9		
	Plasma.	78	73.5			5	2		
14	Whole blood.	94	84		100.5	11	7		13
	Plasma.	104	90		108	3	2		2.5
	Fasting human.								
31	Whole blood.	101	83	82		8.5	13	0.5*	
	Plasma.	126	106	104		0.5	3	−7*	
37	Whole blood.	98				7			
	Plasma.	90				4			
38	Whole blood.	101		87	116	13		7	7
	Plasma.	108		92	106	2		− 1.5	11.5
	Sheep.								
18	Whole blood.		89	85	101		6.5	− 1	18
	Plasma serum.		96.5	85	96		1	− 1.5	13
20	Whole blood.	86.5	79			7.5	4.5		
	Plasma serum.	119.5	116			4.5	0		
	Rabbit.								
25	Whole blood.	236	235	233		9.5	−4	−13*	
	Plasma serum.	283	269			11.5	−2		
	Dog.								
41	Whole blood.	107		97		6		−1	
	Plasma serum.	131		120		6		0.5	

* These low hydrolyzable sugar values were later shown to be partly due to silicate effect. The Folin reagents are so sensitive to silicates that the occurrence of negative hydrolyzable sugar values for normal whole blood may be regarded as an almost certain indication of the unsuitability of the potassium hydroxide solution, even though the paraffin lining may apparently be intact.

by the Benedict (5) and Folin methods. On the other hand, we have noticed similar effects at other times with both blood and urine which suggest the possible existence of a part of the free sugar, during hyperglycemia or glycosuria, in a form readily destroyed by the hydrolytic procedure.

The application of Folin's method to the analysis of plasma hydrolysate frequently gave negative results for hydrolyzable sugar, although similar values for whole blood were apparently satisfactory. The sensitive Folin reagent is evidently affected by substances appearing in the plasma filtrate during hydrolysis, resulting in the production of less color from the same amount of sugar.

TABLE II.

Effect of Added Glucose on Hydrolyzable Sugar Values.

Sample No.	Method.	Mg. per cent of sugar.			
		Free.		Hydrolyzable.	
		Blood.	Blood + 100 mg. per cent glucose.	Blood.	Blood + 100 mg. per cent glucose.
45	Folin-Wu.	104.5	203.5	9.5	12.5
	Folin.	90.5	193	9.5	8.5
	Sumner.	103.5	207	24.5	24
47	Folin-Wu.	84	188	7	13
	Folin.	77	180	3.5	−3
	Sumner.	82	184	16.5	13.5

The large amounts of free and hydrolyzable sugar obtained by the Sumner method remained unexplained. They were not due to the effects of creatinine or uric acid. Neither could a separation of a non-glucose reducing substance be secured by treating filtrates with prepared bone-black.

Although we made extensive use of the newer Benedict reagent (6), no results are reported, because other experiments in this laboratory have demonstrated the impossibility of securing accurate results for hydrolyzable sugar by this method. In the near future the data supporting this criticism will be published. The effect of potassium sulfate upon this reagent, and upon Kingsbury's reagent (8) was negligible. Our interest in the latter reagent awaits the development of a suitable modification for blood analysis.

Hydrolyzable sugar values for blood might be due to an increased reduction of the analytical reagents by free sugar, under the influence of catalyzing substances liberated from blood filtrate during the hydrolysis. If this were true, the addition of glucose to blood filtrate might increase the hydrolyzable sugar values. Actually, the deviations of hydrolyzable sugar under these circumstances are no greater than can be accounted for by the necessary experimental error involved in the four separate determinations (Table II).

When blood filtrate was diluted with an equal volume of distilled water (Table III), the deviations of the hydrolyzable sugar,

TABLE III.

Effect on Sugar Values of Dilution of Blood Filtrate with Equal Volume of Water.

Sample No.	Method.	Mg. per cent of sugar.					
		Free.		Hydrolyzable.		Deviation (on basis of original blood).	
		Original.	Diluted filtrate.	Original.	Diluted filtrate.	Free.	Hydrolyzable.
44	Folin-Wu.	99	52.5	8.5	7	6	5.5
	Folin.	88	47	6.5	2	6	−2.5
	Sumner.	102	50.5	12	16.5	−1	21
47	Folin-Wu.	84	48.5	7	4	13	1
	Folin.	77	39.5	3.5	3	2	2.5
	Sumner.	82	48	16.5	16	14	15.5

as determined by the copper methods, again fell within the limits of analytical accuracy; but the Sumner method indicated great increases as a result of the dilution. Hence, the latter method probably gives the least reliable hydrolyzable sugar values.

Our previous attempts to ascertain the effect of alkali hydrolysis upon the hydrolyzable sugar were unsatisfactory because of the lack of suitable methods for analyzing dilute sugar solutions. Reliable results have now been secured by employing the technique of Folin and Svedberg (10). The feebly colored solutions were diluted to a volume of 10 cc., as suggested by Benedict (6).

Studies of the color proportionality for dilute glucose solutions could not be found in the literature. Accordingly, a series of com-

parisons was made against the 25 mg. standard (2 cc. of 0.0025 per cent glucose solution). Averages of the results are given in Table IV. We found a closer approach to theoretical values for the Folin method than for the Folin-Wu method. The undoubted cause of the lack of proportionality in the latter method is the occurrence of a greater blank. If one assumes the color of the blank to be present in both standard and unknown, corrections would be made thus:

$$\text{Corrected value} = \text{actual value} \times \frac{25 + \text{blank}}{25} - \text{blank (in terms of mg. per cent).}$$

TABLE IV.

Proportionality of Dilute Glucose Solutions.

Method.	Glucose in mg. per cent.			
	Theoretical.	Actual.	Corrected for blank.	Average blank.
Folin-Wu.	25	25	25	4.7
	20	20.7	19.9	
	15	16.2	14.6	
	10	11.8	9.3	
	5	8.1	4.9	
Folin.	25	25	25	1.6
	20	18.9	18.4	
	15	13.7	12.9	
	10	9.2	8.2	
	5	5.1	3.8	

By means of this correction the Folin-Wu values become almost exactly proportional. Similar corrections for Folin's method could not be used to improve proportionality, probably because the so called blank, equivalent to 1.6 mg. per cent of glucose, does not represent actual color produced by reduction of the reagent, but other optical effects. For this reason the blank was never used in our calculations. Sumner's method could not be used in the investigation of very dilute sugar solutions because of the lack of sensitivity of the reagent and the pronounced color of the blank.

No previous mention has been made of an obvious source of

error in the copper methods. The glass cups for the Duboscq colorimeters, frequently used in biological analyses, are surrounded by nickeled cylinders of brass, which come in contact with the analytical solution during pouring or rinsing. The use of these cups is inadvisable because the complex molybdic and tungstic

TABLE V.

Effect of Heating Blood Filtrate, Glycogen, and Glucose Solutions with Alkali.

Sample No.	Method.	Mg. per cent of sugar.			
		Free.		Hydrolyzable.	
Blood.		Original.	After alkali treatment of blood filtrate.	Original.	After alkali treatment of blood filtrate.
43	Folin-Wu.	102	5.5	11.5	7
	Folin.	90.5	3	11.5	6
45	Folin-Wu.	104.5	7.5	9.5	9
	Folin.	90.5	5	9.5	7
47	Folin-Wu.	84	6	7	7
	Folin.	77	7.5	3.5	7.5

Glycogen.	Method.	Free after acid hydrolysis.		Free after acid hydrolysis of glycogen previously treated with alkali.	
			Equivalent to:		Equivalent to:
		mg. per cc.	*mg. per cent*	*mg. per cc.*	*mg. per cent*
1	Folin-Wu.	0.0195	19.5	0.0105	10.5
	Folin.	0.016	16	0.0065	6.5
2	Folin-Wu.	0.027	27	0.0150	15
	Folin.	0.0245	24.5	0.0140	14

Glucose.	Method.	Free.		Free after alkali treatment.	
			Equivalent to:		Equivalent to:
		mg. per cc.	*mg. per cent*	*mg. per cc.*	*mg. per cent*
1	Folin-Wu.	0.100	100	0.0025	2.5
	Folin.	0.100	100	0.0025	2.5
2	Folin-Wu.	0.100	100	0.0045	4.5
	Folin.	0.100	100	0.0025	2.5

acid reagents are readily reduced by metallic nickel and copper, just as they are by cuprous oxide. Careful observation of the interior of the cup, containing such acid reagents, will show a drainage of deeply colored liquid from the metal rim. It is much better to use all-glass cups for sugar analyses, especially when dealing with fermented filtrates, *etc.*

As to the possible nature of the hydrolyzable sugar of blood filtrate, it will be recalled that glycogen, if present in blood, will at least partly escape precipitation with the proteins in the preparation of the filtrate (1). Moreover, the hydrolyzable sugar is largely contained within the formed elements of the blood, as would be expected if the material were glycogen. The results recorded in Table V show the effect of heating 8 cc. portions of blood filtrate, of 0.01 per cent glucose solution, and of 0.005 per cent glycogen[1] solution with 1 cc. of 2.6 N potassium hydroxide solution for 1¼ hours on the water bath. (It was impractical to use the greater concentrations of alkali which are claimed to have no effect on glycogen (11).) After cooling, the mixtures were neutralized with 1 cc. of 2.6 N sulfuric acid. In the case of the glycogen solution it was, of course, necessary to submit the original and alkali-treated solutions to hydrolysis, by the total sugar method, before the sugar was determined. The slight salt effect, due to the increased amounts of potassium sulfate in the hydrolyzed, alkali-treated, analytical samples, was compensated for by using similar amounts of acid and alkali in the standard glucose solutions.

The small amount of color generated by glucose solutions after alkali treatment was very little more than the absolute blank in the Folin method. Therefore, one is justified in claiming the destruction of at least 99 per cent of the glucose. The glycogen solutions, while more resistant to alkali treatment, were markedly affected by the relatively dilute alkali employed. A little less than 50 per cent of the glycogen was destroyed, or changed in such fashion as to prevent the formation of reducing glucose by acid hydrolysis.

The alkali treatment of blood filtrate shows distinctly that the free sugar of blood is not entirely like glucose, since 5 to 7 per cent of the free sugar by the Folin-Wu method, and 3 to 9.5 per cent by the Folin method, remain after the alkali treatment. These values approximate those secured for residual reduction of blood filtrate after fermentation. The hydrolyzable sugar of blood filtrate is usually partly destroyed, as in the case of glycogen, but the losses are quite variable because of the necessarily large experimental error involved. Grossly, the material may be said to resemble glycogen, being at least as resistant to the action of alkali.

[1] C. P. Eastman Kodak Company.

An attempt was made to ascertain whether the sugar, liberated from blood filtrate during the total sugar procedure, resembled glucose in being fermented by yeast. After careful consideration of short period methods of "fermentation" (10, 12), we decided to employ the method of Folin and Svedberg (10). Our work was well under way when Benedict's proposed modification appeared (6). His process is intended chiefly for use with whole blood rather than blood filtrate. Hence it was decided to continue with the Folin-Svedberg method. Washing the yeast, as Somogyi and Benedict advise, is undoubtedly better and would give lower blanks, but the nature of our reported results would hardly be

TABLE VI.

Effect of Fermentation of Hydrolysate on Hydrolyzable Sugar Values.

Sample No.	Method.	Mg. per cent of sugar.			
		Free.		Hydrolyzable.	
		Original.	After fermentation.	Original.	After fermentation.
21	Folin-Wu.	90	10	7	4
	Folin.	81	7	9.5	7
23	Folin-Wu.	96	7	9	8
	Folin.	86.5	3	8.5	8
24	Folin-Wu.	88	8	10	3.5
	Folin.	74	4	6	4.5
36	Folin-Wu.	93	8.5	7.5	5
	Folin.	78	5.5	−1*	3

* Silicate effect.

altered. In a very recent paper, Somogyi (13) has recommended a new procedure for filtrate. At a later date we intend to repeat some of our experiments, using this technique.

The blanks for the yeast, to be used in correcting the free sugar values, were determined upon freshly prepared 0.01 per cent glucose standard. Similar blanks for the total sugar determinations were made on the glucose standard plus the requisite amount of potassium sulfate (added as alkali and acid). In the presence of the salt, yeast apparently contributed slightly more reducing material, the average increase being 1.5 mg. per cent of sugar for the series of experiments.

The results of these experiments (Table VI) indicate that a large proportion of the hydrolyzed sugar is not "fermented." It would be more accurate to say that it is neither absorbed nor adsorbed by the yeast. The results are sufficiently variable to cast doubt upon the validity of the fermentation test under these circumstances.

As a check upon the foregoing experiments, glycogen solution was also fermented (Table VII). In Experiments 1 and 2 a 0.005 per cent, freshly prepared, unpreserved solution of glycogen was used; and in Experiment 3 a solution of 10 times this strength. The solutions were first hydrolyzed by the total sugar method and the fermentation carried out as previously described,

TABLE VII.

Effect of Fermentation on Glycogen Hydrolysate.

Sample No.	Method.	Free sugar.			
		Before fermentation.		After fermentation.	
			Equivalent to:		Equivalent to:
		mg. per cc.	*mg. per cent*	*mg. per cc.*	*mg. per cent*
1	Folin-Wu.	0.017	17	0.0075	7.5
	Folin.	0.0175	17.5	0.004	4
2	Folin-Wu.	0.020	20	0.005	5
	Folin.	0.0165	16.5	0.0025	2.5
3	Folin-Wu.	0.192	192	0.057	57
	Folin.	0.159	159	0.069	69

except that the blanks for the yeast were determined on glycogen solutions rather than on glucose solutions. Corrections have been made for the small increased yeast blank due to the salt effect. The results show that a variable portion, but approximately one-third, of the reducing sugar set free by the hydrolysis of glycogen is not removed by the yeast. Therefore the residues of reducing sugar from fermented hydrolysates of blood filtrates do not constitute evidence against the possibility of the hydrolyzable sugar of blood being glycogen. The failure of yeast to remove all of the hydrolyzed glycogen complicates the interpretation of the non-fermentable residues of blood sugar.

A further example of the complex conditions to be encountered in fermented blood filtrates is given by several experiments in

which fermentation preceded hydrolysis (Table VIII). The purpose of these experiments was to determine whether the hydrolyzable sugar could be removed from the filtrate in a similar fashion to glucose. It was not possible to secure a definite answer to this question. After the calculations had been made for Sample 44, it was evident that some hydrolyzable sugar had been extracted from the yeast by the blood filtrate. In the two subsequent experiments, in addition to the customary blanks for the yeast, 0.01 per cent freshly prepared solution of glucose was fermented, and the filtrate of this mixture hydrolyzed by the total sugar method. Large amounts of hydrolyzable sugar were found to be present in these solutions. Even after the large blanks had been

TABLE VIII.

Effect of Fermentation of the Filtrate before Hydrolysis.

Sample No.	Method.	Mg. per cent of sugar.					
		Free.		Hydrolyzable.		Yeast blank.	Increases in blanks from hydrolysis (equivalent to hydrolyzable sugar).
		Original.	After fermentation.	Original.	After fermentation.		
44	Folin-Wu.	99	9.5	8.5	18.5	5.8	
	Folin.	88	4	6.5	16.5	3.5	
46	Folin-Wu.	90	12	10	12	9.8	20.4
	Folin.	83	6	3	11	8.3	18
48	Folin-Wu.	94	18	10	23	13.1	26.7
	Folin.	77	3	6	22	10.5	11.2

subtracted, more than the original amounts of hydrolyzable sugar were present in the fermented blood filtrates. These results confirm the validity of Somogyi's recommendation that the yeast be thoroughly washed.

In Table IX we present further data on the hydrolyzable sugar of human whole blood. We have also included the average values for the hydrolyzable sugar of normal whole blood and plasma.

The leucemic blood (Sample 49), obtained through the courtesy of Dr. Hugh G. Jeter of the University Hospital, had a white cell count of 130,000, and contained more hydrolyzable sugar than normal blood. While approximately 20 times the normal number

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TABLE IX.

Hydrolyzable Sugar of Human Whole Blood.

Sample No.	Mg. per cent of sugar.							
	Free.				Hydrolyzable.			
	Folin-Wu.	Benedict.	Folin.	Sumner.	Folin-Wu.	Benedict.	Folin.	Sumner.
2	80				9			
3	98	89		91.5	6	8		10.5
4	88.5	88.5			10	9		
6	87	86			6	5		
7	98	91			12.5	17		
8	98	91	83		12.5	7	13	
9	82.5	77	71		15	9	10	
10	98	91	83		16	17	13	
13	73.5	70.5			14	9		
14	94	84		100.5	11	7		13
19	91			107	7			1
21	90		81		7		9.5	
23	96		86.5		9		8.5	
24	88		74		10		6	
31	101	83	82		8.5	13	0.5*	
33	99				11.5			
34	76		63		9		1*	
35	95		84		6.5		−1*	
36	93		78		7.5		−1*	
37	98		85		7		0*	
38	101		87	116	13		7	7
39	81		70	81	7.5		2	15
40	114.5		103	126	9.5		5	9.5
42	99		88	99	10		9.5	19
43	102		90.5	102.5	11.5		11.5	16.5
44	99		88	102	8.5		6.5	12
45	104.5		90.5	103.5	9.5		9.5	24.5
46	90		83	95	10		3	15
47	84		77	82	7		3.5	16.5
48	94		77	100.5	10		6	11.5
49 (leucemia).	68		54	62	12		15.5	36.5
Average normal values.								
Whole blood.					10	11	8	14
Plasma.					3	3	Indeterminate.	Insufficient data.

* Values for hydrolyzable sugar made with alkali which had a silica effect. The reported values are approximately 9 mg. per cent too low.

of white cells were present the hydrolyzable sugar was but twice the normal amount by the Folin method, and only slightly increased by the Folin-Wu method. If the analyses of glycogen in mammalian leucocytes by de Haan (14) be taken as a basis for calculation, Sample 49 should have contained approximately 45 mg. per cent more glycogen than normal samples because of the increased white cell count. This amount of glycogen would increase the hydrolyzable sugar by approximately 20 mg. per cent of glucose. Some glycogen was undoubtedly lost in the protein precipitate (1), and pathologic white blood cells might contain less glycogen than normal cells. According to de Haan's findings normal blood should contain approximately 2 to 4 mg. per cent of glycogen, of white cell origin. This amount of glycogen would give, by our total sugar method, a hydrolyzable sugar value of 1 to 2 mg. per cent of glucose. Normal blood contains 5 to 10 times this amount of hydrolyzable sugar, the greater part of which cannot at present be traced to a leucocytic origin.

Since glycogen has never been detected in erythrocytes, we conclude that the greater portion of the hydrolyzable sugar of blood is not glycogen, or else true analytical values for the glycogen of blood cells have not been attained. Perhaps the use of concentrated alkali in the separation of small amounts of glycogen leads to some destruction. We hope by isolation experiments to be able to secure added data on the nature of the hydrolyzable sugar of blood. Recently Fontes and Thivolle (15) and Bigwood and Wuillot (16) have published data concerning the nature of "combined sugar" of blood filtrates, but the different technique employed prevents direct comparison with our results.

SUMMARY.

Further investigations concerning the suitability of the total blood sugar method are reported. Evidence is presented to show that the hydrolyzable sugar of blood actually exists, and is not due to analytical errors. Values are given for the hydrolyzable sugar of plasma. Folin's method cannot be used for these plasma determinations. Essentials for the analysis of dilute sugar solutions are discussed. The effects of fermentation and of alkali

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hydrolysis upon the hydrolyzable sugar indicate that it resembles glycogen, but the latter has never been found in sufficient quantities in normal blood to account for more than a small part of the hydrolyzable sugar (14, 17–20). A sample of leucemic blood contained more hydrolyzable sugar, but not enough to indicate the white cells as the chief source of this material.

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CUTANEOUS AND VENOUS BLOOD SUGAR CURVES.

I. IN NORMAL INDIVIDUALS AFTER INSULIN AND IN LIVER DISEASE.

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In an attempt to define more accurately the nature of the disturbances of carbohydrate metabolism in mild diabetics and persons with benign glycosuria, the authors have investigated the arterial (cutaneous) and venous blood sugar curves of a variety of subjects after the administration of carbohydrate food.

HISTORICAL.

The fact that the concentration of glucose in systemic arterial blood exceeds that in the systemic venous blood was first demonstrated by Chauveau (1) in 1856, before Claude Bernard had discovered glycogen or was even willing to admit the presence of glucose in systemic blood. Perhaps for this very reason the difference between arterial and venous blood was overlooked by most subsequent observers, an oversight that has had a definite influence on the history of carbohydrate metabolism studies and has led to many unnecessary controversies.

Bang (2) in his important monograph, published in 1913 and, unfortunately, never translated into English, was aware of the arterial-venous difference and was influenced by its existence. In this work he presented a method for the determination of sugar in 0.2 cc. of blood or less. Almost simultaneously Lewis and Benedict (3), in this country, described a method suitable for the analysis of about 2 cc. of blood. These two methods for the first time made blood sugar determinations practical clinical procedures. Bang's procedure was generally adopted in Europe, and became the standard on which other techniques were modeled. It was suitable for the analysis of such small amounts of blood as could be obtained by puncture of the skin of the finger or the ear. Benedict's method was as generally accepted in America and subsequent methods developed on this side of the Atlantic followed Benedict's lead in requiring 1 or 2 cc. of blood or its equivalent, rendering them applicable only to the study of venous blood.

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Hagedorn (4) and Foster (5) have shown that cutaneous blood is identical with arterial blood as far as its sugar content is concerned. It therefore follows that European investigators have in general examined arterial blood, while Americans have examined venous blood in their clinical studies.

Although Bang (2) was aware of the difference between arterial and venous blood and, presumably, was influenced by this knowledge, there is little evidence that subsequent workers chose their methods of study with any consciousness that such a difference existed until Hagedorn (4) published his investigations of the subject in 1920. Folin and Berglund (6), indeed, offered as an explanation of the greater alimentary hyperglycemia found by European workers, the theory that puncture of the finger or ear caused greater pain than venous puncture, and, therefore, greater nervousness. After the publication of Hagedorn's studies Scandinavian investigators, at least, began to appreciate the importance of distinguishing between cutaneous and venous blood. It was not, however, until 1923 that the distinction was recognized in this country by Foster (5), who confirmed in every respect the observations of Chauveau (1) and Hagedorn (4). Since then Holst (7), Lawrence (8), Lundsgaard and Holbøll (9), and Rabinowitch (10) have made studies of the alimentary arterial venous blood sugar difference in normal persons and in diabetics.

All workers agree that in the postabsorptive state the concentrations of sugar in arterial and venous blood are identical. After the ingestion of carbohydrate both rise. The rise of the venous curve is checked, however, before that of the arterial and falls more rapidly. At a variable period after the meal the venous sugar sinks not only to, but actually below, the initial postabsorptive level. This is the postalimentary hypoglycemia, first described by Bang (2). The cutaneous curve reaches the initial level more slowly than does the venous curve, but, like the latter, eventually tends to fall slightly below it. The two curves then converge at the fasting level. Holst (7), Lawrence (8), Lundsgaard and Holbøll (9), and Rabinowitch (10) have all found that in the severe diabetic the alimentary arterial-venous difference may be absent or greatly diminished. Lundsgaard and Holbøll (9) claim that the difference can be restored to the diabetic by the administration of insulin.

Cori and Cori (11) have, by a clever series of experiments, demonstrated the mechanisms which determine the form of the alimentary blood sugar curve and its variations under different conditions. At all times glucose is being absorbed from the blood by the muscles and other tissues to be stored as glycogen, which is, in turn, continually decomposed to lactic acid and oxidized. The rate of removal is, in the resting, fasting state, however, too small to be readily distinguished. The blood sugar is as continuously replenished from the hepatic stores of glycogen, which are obtained from ingested preformed glucose or by the transformation in the liver of other carbohydrates or protein. The initial rise of arterial and venous blood sugar after the ingestion of glucose is an expression of the absorption of sugar from the alimentary tract. The subsequent reduction of the blood

sugar to the normal level is the result of two factors: (1) the removal of sugar by the liver to form glycogen; (2) the removal of sugar by the tissues, and especially muscle, for the formation of tissue glycogen and for combustion. It is the latter process that is evidenced in the arterial-venous difference. Insulin appears to exert its chief effect in promoting removal and consumption of sugar by the tissues.

Because the blood sugar begins to fall while absorption of carbohydrate is still at its height and because alimentary hyperglycemia is eventually succeeded by a period of hypoglycemia, it may be inferred that the mechanisms for the removal of sugar from the blood and its disposal have a certain inertia. They are activated to their full extent only after the blood sugar has risen measurably above the normal level and are not again checked until it has fallen distinctly below this level.

Investigative Procedure.

For the analysis of blood a micro modification of Benedict's 1925 (12) procedure was employed. Earlier attempts to adapt the method of Folin and Wu (13) to micro analysis were not entirely successful, although it has been so employed by Foster (5) and others. Benedict's method has a definite advantage in the fact that the color developed is more intense. His proposal that benzene be vaporized in the tubes during the reduction procedure proved entirely successful in our hands and obviated the necessity of using special tubes. When the method began to be applied more generally, however, it proved simpler to have tubes constructed which were especially adapted to the micro procedure. These will be described below.

In 1926 Benedict (14) modified his method to meet certain objections. He pointed out that bisulfite, which had been used in the copper solution was not a salt of constant composition and substituted sulfite. He also added ammonium salts to keep the reduced copper in solution. Finally he increased the acidity and the specific gravity of the arsenophosphotungstate color reagent by the addition of HCl and NaCl. An early attempt to adapt this method directly to micro analysis proved unsuccessful because of the frequent appearance of precipitates. No such difficulty was encountered when it was employed for the analysis of the usual volumes of blood filtrate. More recently, with the smaller tubes no precipitation has occurred. The 1925 method had, however, proved so satisfactory that it seemed best to continue it.

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On examination of the results of all the studies together, an interesting fact appears. The method has been employed by all four of the authors in different phases of the investigation and by three technicians in the same laboratory. It has been found by all to check perfectly with the similar macro procedure at all times. As it was employed glucose solutions equivalent to filtrates from bloods containing glucose varying from 50 to 400 mg. per 100 cc. can be compared without error in the colorimeter. When the results of the various studies are compared, it is found that the normal values obtained in 1926 are all consistently lower than those of 1927 and 1928. The difference was first ascribed to the fact that the subjects of the 1927 series were given glucose, while those of 1926 were given mixed meals. The fact that fasting values, as well as those after food, were low was overlooked.

The only explanation that can be offered for the discrepancy is that different reagents were used for the first series. Different supplies of bisulfite may have been responsible. When Benedict originally proposed the method he pointed out that it gave values consistently lower and probably more correct than the Folin-Wu procedure. This we verified in the 1926 series. In the later series, however, the values obtained are quite as high as those usually obtained by the Folin-Wu method.

Analytical Procedure.

In a pipette carefully calibrated *to contain* 0.2 cc. blood was secured from the ear or finger tip of the subject. Venous blood was withdrawn by means of a small hypodermic syringe, transferred at once to a clean glass container, and thence drawn into the pipette. The blood thus obtained is immediately delivered into 1.6 cc. of N/12 H_2SO_4 in a conical centrifuge tube and the pipette carefully washed out by drawing the acid into it two or three times. The blood is well mixed with the acid by means of a small footed stirring rod. When it is completely laked 0.2 cc. of 10 per cent sodium tungstate is added, the whole is thoroughly mixed with the stirring rod, and, after standing a few minutes is centrifuged. Sufficient protein-free supernatant fluid for duplicate blood sugar determinations can be secured from 0.2 cc. of blood treated in this manner.

0.5 cc. of the supernatant fluid is transferred to a Folin-Wu

blood sugar tube, an ordinary test-tube of moderate diameter, calibrated at 6.25, 12.5, and 25.0 cc., or, best of all, one of the special tubes described below. In a similar tube is placed 0.5 cc. of a standard glucose solution containing 0.1 gm. of glucose per liter. Standard and unknown are thereafter treated alike. To each is added 0.5 cc. of the Benedict copper solution. Unless the special tube is used, 4 drops of benzene must then be added and the tubes stoppered with cotton. They are then placed in boiling water, where they are allowed to stand for 5 minutes. At the end of this time they are cooled in cold water for 2 minutes. After this 0.5 cc. of the arsenophosphotungstate solution is introduced. The mixture is allowed to stand 10 minutes longer to insure complete solution of the reduced copper. In some instances this must be aided by agitation of the tube.

At the end of this time standard and unknown are diluted and compared in a colorimeter. The colors of standard and unknown should not differ by more than 30 per cent. It is, therefore, necessary either to employ several standards of different strengths or to use a combination of dilution and comparison colorimetry as advocated by Rothberg and Evans (15). If the blood sugar is less than 100 mg. per 100 cc. the color is so faint as to make colorimetric comparison difficult, even if it is diluted only to 12.5 cc. It is, therefore, advisable, if Rothberg and Evans or Folin-Wu tubes are used to mark them at 6.25 cc.

Special tubes have been devised which greatly facilitate the procedure. These are similar to the Rothberg and Evans (15) modification of the Folin-Wu tube, but smaller. They are so made that when 1 cc. of fluid is introduced, the upper level of the liquid lies in the constricted portion of the tube which must not exceed 6 mm. in diameter. The tubes are graduated at 1.25 cc. intervals from 6.25 to 25.0 cc. With these tubes standard and unknown can be diluted till their colors are a convenient depth and almost match before comparison in the colorimeter. By this combination of dilution and comparison colorimetry bloods varying in sugar concentration from 50 to 400 mg. per 100 cc. can be analyzed with a single standard without error. The use of benzene to prevent reoxidation of the reduced copper is also unnecessary.

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Calculation of Results.

$\frac{100 SV_sQ}{RV_s}$ = mg. of glucose per 100 cc. of blood, where S and R = readings of standard and unknown respectively; V_s and V_r = the volume in cc. to which standard and unknown, respectively, were finally diluted; Q = the strength of the standard solution in gm. of glucose per liter.

Treatment of Material.

In all experiments on alimentary hyperglycemia, blood was secured in the morning in the postabsorptive state, at least 12 hours after the last preceding meal. As soon as possible after the preliminary sugar determination, the subject was given a known dose of glucose dissolved in the diluted juice of an orange or a lemon. After this the blood sugar was again determined at 30 minute intervals for from 90 to 120 minutes. When insulin was given without food the blood sugar was determined before and at 30 minute intervals after insulin injection.

An attempt was made, in every case, to secure both venous and arterial samples as nearly as possible simultaneously. The venous sample was usually taken first. The total time spent in withdrawing the two bloods seldom exceeded 3 minutes.

Urines from all subjects were tested for sugar by Benedict's qualitative test before and after each experiment. None of the normal subjects showed glycosuria.

Normal Alimentary Reaction of Arterial and Venous Blood.

The data obtained from normal individuals are collected together in Table I. In all twenty-four studies were made on twenty normal adults (medical students, interns, and laboratory workers). The ten of the 1927 series were studied for 2 hours after a meal consisting of 50 gm. of glucose dissolved in 200 cc. of water with the juice of an orange or lemon. The five of the 1926 series were studied seven times for 1.5 hours after mixed meals containing 75 to 115 gm. of carbohydrate. In two additional experiments one of the subjects received on one occasion 75 gm., on another 150 gm. of glucose. The five of the 1928 series were studied for 1.5 hours after mixed meals containing from 70 to 100 gm. of carbohydrate.

These various procedures were chosen because the individual

series were intended to serve as controls for different types of experiments. As has been pointed out in the discussion of analytical procedure, in the 1926 series, as a whole, all values lie distinctly below those of other years. This is probably due neither to technical errors nor to peculiarities of individuals examined during the year 1926, but to differences in the chemicals (probably the bisulfite) used in the preparation of the reagents. Separate controls were made at intervals in each series to prove that intensity of color varied directly with sugar concentration. The only possible

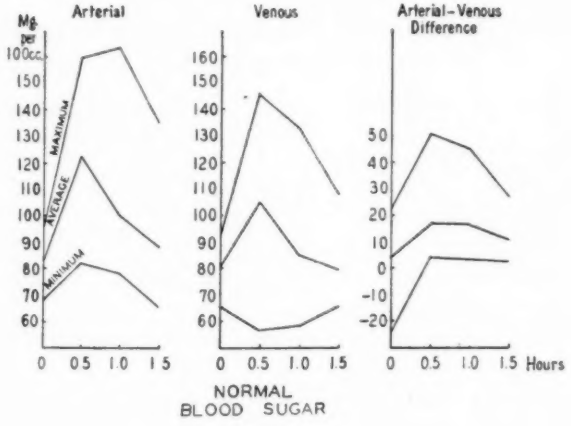

FIG. 1.

conclusion then, is that the 1926 reagents were less susceptible to non-glucose reducing substances than were subsequent reagents. If the 1926 values are all increased by 25 mg. per 100 cc. they agree well with those of other years both in fasting level and in degree and duration of hyperglycemic reaction.

Examination and comparison of all the series after the 1926 values have been increased by 25 mg. show no demonstrable difference in the hyperglycemic reaction after 50 gm. of glucose and that after a mixed breakfast. The variability of the reaction after either type of meal is too great to permit one to make any clear distinction.

TABLE I.

Blood Sugar Values of Normal Individuals.

Mg. per 100 cc.

Subject No.	Fasting.			Time after meal.												Meal.
				30 min.			60 min.			90 min.			120 min.			
	Cutaneous.	Venous.	Difference.	Cutaneous.	Venous.	Difference.	Cutaneous.	Venous.	Difference.	Cutaneous.	Venous.	Difference.	Cutaneous.	Venous.	Difference.	
1917																Glucose.
1	78	70	8	121	107	14	74	75	−1	86	73	13	87	70	17	50 gm.
2	74	71	3	129	115	14	97	83	14	67	66	1	68	66	2	50 "
3	73	74	−1	108	78	30	96	97	−1	94	70	24	84	73	11	50 "
4	94	89	5	125	101	24	107	89	18	104	86	18	88	81	7	50 "
5	85	78	7	120	112	8	106	103	3	102	94	18	81	88	−7	50 "
6	68	66	2	98	88	10	105	89	16	77	98	−21	85	79	6	50 "
7	108*	74	34	127	112	15	83	63	20	81	71	10	76	62	14	50 "
8	95†	89	6	128	109	19	144	128	16	169	132	37	124	125	−1	50 "
9	93	71	22	158	108	50	102	72	30	84	70	14	77	66	11	50 "
10	85	83	2	149	128	21	92	78	14	88	72	16	85	77	8	50 "
Maximum.	108	89	34	158	128	50	144	128	30	169	132	37	124	125	17	
Minimum.	68	66	−1	98	78	8	74	63	−1	67	66	−21	68	62	−7	
Average.	85	77	9	126	106	21	101	88	13	95	83	12	86	79	7	

1920													Mixed meal.
1 a	60	59	1	97	93	4	74	52	22	62	52	10	Carbohydrate. 102 gm.

1920													
													Mixed meal. Carbohydrate.
1 a	60	59	1	97	93	4	74	52	22	62	52	10	102 gm.
b	56	64	−8	113	103	10	85	75	10	54	45	9	102 "
c	59	58	1	103	83	20	91	46	45	58	33	25	75 " (glucose).
d	56	56	0	81	70	11	77	66	11	72	61	11	170 " (150 gm. glucose).
2 a	51	50	1	59	53	6	55	52	3	40	52	−12	74 "
b	63	63	0	105	73	32	61	49	12	41	49	−8	74 "
3	68	65	3	96	92	4	61	46	15	52	42	10	96 "
4	56	56	0	78	66	12	76	64	12	43	42	1	116 "
5	70	58	12	102	90	12	95	75	20	98	78	20	101 "
Maximum.	70	65	12	113	103	32	95	75	45	98	78	25	
Minimum.	51	50	−8	59	53	4	51	46	3	40	42	−12	
Average.	60	59	1	93	80	12	75	58	17	58	50	7	
	85	84		118	105		100	83		83	75		
1918													
													Mixed meal. Carbohydrate.
1	75	78	−3	112	89	23	92	81	11				85 gm.
2	69	93	−24	104	97	7	90	72	18	83	74	11	97 "
3	90	84	14	77	57	20	85	59	26	96	75	21	73 "
4	97	90	7	160	146	14	163	132	31	135	108	27	80 "
5	83	77	6	149	135	14	132	118	14	103	98	5	67 "
Maximum.	97	93	14	160	146	23	163	132	31	135	108	27	
Minimum.	75	77	−24	77	57	7	85	59	11	83	74	5	
Average.	83	84	0	120	105	16	112	92	20	104	94	16	

* Some trouble and delay was encountered in securing the venous blood. This may have caused sufficient nervousness to disturb the blood sugar results.

† This patient felt faint just before the test.

Fig. 1 represents graphically the extent of variation of arterial and venous blood sugar changes in the whole series and the differences between the two. In this and subsequent figures 25 mg. have been added to all the 1926 values. As it is not absolute values but changes resulting from disease or other factors that are under consideration, such correction seems justified. Actual determined values are given in all tables.

In the fasting state the arterial venous difference was less than 10 in nineteen out of twenty-four determinations. In nine instances a difference of more than 5 mg. was observed. In only four of these did it exceed 8 mg. Even at rest in the fasting state the removal of sugar by the tissues can apparently become accelerated to a point where it is easily perceptible. At times it may become quite striking. The value of 34 obtained on Subject 7 of the 1927 series should probably be discounted because some difficulty was experienced in securing the first venous sample. 15 minutes altogether were consumed in taking both venous and arterial blood and the subject experienced considerable pain. No such factors, however, were active in the experiment on Subject 9 of this series or Subject 3 of the 1928 series in which differences of 22 and 14 respectively were observed.

In two instances negative (arterial < venous) differences of significant magnitude (−8 and −24) occurred. Such negative differences will be encountered in other connections with sufficient frequency to make it evident, even if duplicates and controls had not been carefully carried out, that they are not due to technical errors. They have also been reported by others. The obvious explanation for them is that at times sugar is given off by the tissues to the blood, an idea that is contrary to physiological conceptions of carbohydrate metabolism. Bollman, Mann, and Magath (16) have shown that glycogen of muscles is not available for the restoration of the blood sugar in the hypoglycemia following removal of the liver. Cori and Cori (11) have also been unable to demonstrate such a reversal of the normal sugar current.

Folin and associates (17) have suggested that sugar may be temporarily absorbed and held by tissues in the free state, to be liberated into the blood later for distribution. It may be such sugar that is evidencing itself in these experiments and not sugar derived from tissue glycogen. However, there is no apparent reason why tissues in the fasting state should contain an excess

of free sugar. General hemoconcentration could hardly explain such large differences. On the whole they would rather seem to indicate that, under certain conditions, some part of the carbohydrate in tissues can be mobilized into the blood.

In most instances both arterial and venous sugars rise quite rapidly, reaching their maxima in half an hour, and are already on the descent at the end of an hour. The height to which they rise is variable, a variation that is probably partly the result of the procedure employed. Hansen (18), Hatlehol (19), and others who have studied cutaneous blood sugar at frequent intervals have shown that the peak of the alimentary curve is quite sharp and occurs at a variable interval after meals, usually less than an hour. If the peak is detected in any of the curves here presented it is due to a mere coincidence. One can only conclude from these studies that the chances of detecting the maximum rise are greater 30 minutes than they are 60 minutes after a meal. In four instances the values at 60 and 90 minutes were higher than those at 30. The subject of one of these experiments, No. 8, 1927, was very nervous and almost fainted when the first blood sample was taken. His curve is the highest and most prolonged in the series, attaining a maximum of 169 only after 90 minutes and remaining as high as 124 at the end of 2 hours. Although he seemed normal and his nervousness passed off almost immediately, it is doubtful whether this curve should be included in the normal group. The curve for Subject 4 of the 1928 series, another delayed curve, also attained an unusual height. Both of the other delayed curves, Subject 6, 1927, and Subject 3, 1928, showed unusually small fluctuations, permitting little significance to be attached to the delay.

In no experiment did the venous sugar rise as high as the arterial, although significant negative differences were observed in three instances. The maximal, minimal, and average rises of arterial and venous sugars and the differences between them were:

	Maximum. *mg. per 100 cc.*	Minimum. *mg. per 100 cc.*	Average. *mg. per 100 cc.*
Arterial rise	74	6	41
Venous rise, actual	58	−7	27
" " above fasting arterial	52	−15	23
Arterial-venous difference	50	6	22
Increase of difference over fasting	44	−14	18

The arterial blood sugar was in every experiment higher after the meal than in the fasting state; the rises were, however, in some instances very slight. The venous sugar always rose less than the arterial and, when the rise of the latter was minimal, the venous sugar occasionally fell so that it was lower at the 30 and 60 minute periods than in the fasting state. In every case, no matter how little the arterial sugar rose, a definite positive arterial-venous difference was established at the 30 or 60 minute interval or both. This difference was greater than the fasting difference in every experiment but one, Subject 7, 1927, to which reference has been made before. The difference varied from 6 to 50 mg. and, in all but two experiments, was maximal at the 30 or 60 minute points. The maximal difference occurred with equal frequency at the end of 30 or 60 minutes. It coincided with the highest point on the curve in seventeen of the twenty-four experiments and appeared when the curve was falling in six. It was less than 5 mg. twice at 30 minutes and four times at 60 minutes.

It is, then, possible in all normal individuals after a meal of 50 gm. of glucose or a mixed meal containing carbohydrate, to detect after 30 or 60 minutes or both a positive arterial-venous difference of significant magnitude, an expression of the activity of the tissues in removing sugar from the blood. This may, however, be overlooked if both 30 and 60 minute points are not determined.

Holst (7) states that the magnitude of the difference varies directly with the degree of hyperglycemia produced. It has already been pointed out that the experiments here reported do not measure the height of the blood sugar rise. There is, however, no definite correlation in these experiments between the degree of hyperglycemia observed and the magnitude of the arterial-venous difference.

In the ten experiments of the 1927 series the cutaneous blood sugar had returned to or below the original postabsorptive level at the end of 60 minutes twice, within 90 minutes three times, and twice within 120 minutes. In two other experiments, although it had not fallen to the original level, it had dropped below the upper limits of the normal blood sugar range. In only one experiment did it remain definitely elevated after 2 hours, Subject 7, the one in which the subject became faint, to which reference has been made above. The results of the other two series are not

dissimilar, although the studies were not carried beyond 90 minutes and, therefore, restoration of the normal was less frequently observed. Altogether seventeen of the twenty-four curves returned to normal within 90 minutes. Nineteen of the venous curves had fallen to the resting postabsorptive value in the same interval.

At the 90 minute period negative arterial-venous differences of considerable magnitude, 8 to 21 mg., were observed three times. On two of these occasions, Experiments 2 a and b, 1926, they were associated with definite arterial hypoglycemia. In the third, Experiment 6, 1927, the arterial sugar had returned within the normal fasting limits. These results suggest that the negative differences may represent a reaction to the postalimentary hypoglycemia which results from overstimulation of the processes which remove excess glucose from the blood stream.

Effect of Insulin on Fasting (Postabsorptive) Blood Sugar of Normal Individuals.

If insulin acts chiefly by accelerating the removal of sugar from blood by the tissues and may even, as Cori and Cori (11) have demonstrated, mobilize glycogen from the liver to the muscles by way of the blood, in which it exists as glucose, it should be possible to demonstrate a positive arterial-venous difference in the fasting individual during the development of insulin hypoglycemia. That such a difference is demonstrable is shown in Table II, which gives the effect of 10 units of insulin on the cutaneous and venous blood sugars of five presumably normal individuals in the postabsorptive state. Blood sugar values were determined at 30, 60, and 90 minutes after insulin injection.

In four of the five subjects the lowest blood sugar level was observed after 60 minutes. In DE the maximum effect occurred earlier. Three of the five presented distinct positive arterial-venous differences after either 30 or 60 minutes or both, coinciding with the minimal blood sugar level. In S no appreciable difference was detected. One cannot argue from this, however, that it did not occur between observations when the transient character of the difference in the others is taken into consideration.

The last subject, RN, showed a most peculiar reaction. At the

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30 minute period, while the blood sugar was falling, a small negative difference, which had existed in the fasting state, became exaggerated. This is difficult to explain. At the lowest observed point, 60 minutes, just before he developed symptoms of insulin shock, a hardly significant positive difference had developed. That the tissues were capable of absorbing carbohydrate is demonstrated by the large difference which appeared after this subject had received carbohydrate to relieve the shock symptoms. At

TABLE II.

Fasting Blood Sugar Values of Normal Individuals before and after Insulin.

Mg. per 100 cc.

Subject.	Before insulin.			After insulin.									
				30 min.			60 min.			90 min.			
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Insulin.
													units
K	99	96	3	82	73	10	61	50	11	71	66	5	10
S	79	77	2	69	69	0	51	48	3	59	55	4	10
DE	83	81	2	58	41	17	57	71	−14	45	66	−21	10
R	78	74	4	74	70	4	65	38	27	59	58	1	10
RN	89	93	−4	68	74	−6	54	50	4	149*	124*	25	10
Average.	85	82	3	71	63	8	59	52	7	59	61	−3	

* At the 60 minute period the subject developed definite symptoms of insulin shock. The last blood examination was made at the end of 120 minutes, after he had received an orange, an apple, a small dose of glucose, and his regular breakfast. These values have been omitted in calculating the averages.

times he had glycosuria and showed abnormal reactions to carbohydrate on several occasions.

One subject, DE, developed large negative differences during his recovery period, suggesting again that the tissues may give up glucose to the blood in the reaction to hypoglycemia.

Effect of Diseases of the Liver on Alimentary Hyperglycemia.

If the sugar which enters the blood after the ingestion of glucose is removed both by the tissues and by the liver, one should expect

very definite abnormalities in the curve of alimentary glycemia of persons who have liver disease. Because such individuals partially or totally lack one of the mechanisms that reduces hyperglycemia, hepatic glycogen formation, the curve would presumably be excessively high or prolonged. On the other hand, the power

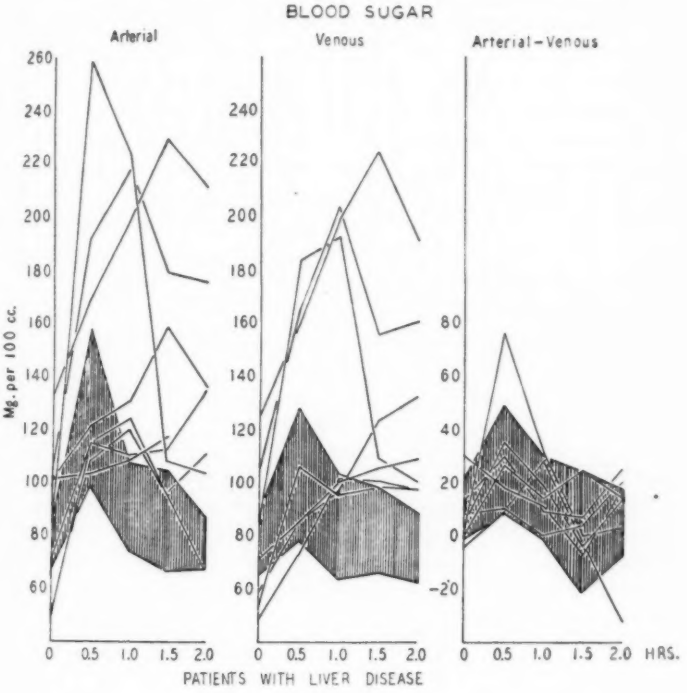

FIG. 2.

of the tissues to remove glucose remaining intact, the arterial-venous difference should manifest itself in the normal manner.

Table III and Fig. 2 show that the facts accord well with expectation. Cutaneous and venous blood sugar determinations were made before and for 2 hours after 50 gm. of glucose on seven

TABLE III.

Blood Sugar Values in Liver Disease. Reaction to 50 Gm. of Glucose.

Mg. per 100 cc.

Subject.	Fasting.			Time after meal. 30 min.			60 min.			90 min.			120 min.			Diagnosis.
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	
HM	52	48	4	108	73	35	120	101	19	96	100	−4	111	97	14	Catarrhal jaundice.
AL	49	53	−4	115	106	9	123	95	28	96	98	−2	65	97	−32	" "
CP*	76	69	7	69	67	2	80	74	6	84	82	2	91	94	−3	Carcinoma of stomach with metastases to liver. Pyloric obstruction. Jaundice, before and after gastroenterostomy.
	71	58	13	115	85	30	109	100	9	112	105	7	134	109	25	
JS	101	91	10	121	†		130			158			136			Portal cirrhosis. Ascites and jaundice.
JP	133	125	8	169	159	10	198	198	0	228	224	4	210	190	20	Portal cirrhosis. No ascites nor jaundice.
M	105	105	0	192	165	27	217	203	14	179	155	24	174	160	14	Toxic hepatitis and cholangitis.
LW	85	84	1	259	183	76	224	192	32	108	108	0	103	100	3	Arsenical hepatitis.
FM	101	71	30	103	85	18	107	96	11	116	123	−7	150	132	18	Pylephlebitis (?). Jaundice following appendectomy.
Maximum.	133	125	30	259	183	76	224	203	32	228	224	24	174	160	24	
Minimum.	49	48	−4	103	73	2	109	95	0	96	98	−7	65	97	−32	
Average.	85	78	7	159	122	30	157	141	18	134	130	3	134	126	9	

* The failure to develop hyperglycemia in this case is probably due to non-absorption of glucose from the gastrointestinal tract because of pyloric obstruction. This observation is omitted in the calculation of averages.

† Venous blood could not be obtained because of the absence of suitable veins.

XUM

patients with diseases affecting the liver. From the fourth, JS, only a cutaneous curve was secured because venous blood could not be obtained. Three of the eight blood sugar curves rose higher than any of those of normal subjects. The excessive hyperglycemia was evident in both cutaneous and venous bloods. Furthermore, seven of the eight arterial curves and all of the venous curves at the end of 2 hours lay above the normal range defined by the comparable experiments of 1927 (see Fig. 1). In the figure the limits of variations of nine of the normal curves (the curve of Subject 8, who almost fainted, is omitted) of the 1927 series are indicated by the shaded areas.[1] The curves of the subjects with liver disease are indicated by the superimposed lines. In spite of the abnormally high and prolonged hyperglycemia, in every case a very distinct positive arterial-venous difference was demonstrated, usually maximal at the 30 minute period.

It is, therefore, possible in patients with severe liver damage to demonstrate in the curve of alimentary glycemia impairment of hepatic glycogen formation without disturbance of the process of sugar removal by the tissues. The deviation from the normal in some of these cases is so small and the range of normal variation so large, that it is doubtful whether the determination of such curves will aid in the diagnosis of hepatic disease.

The first study on CP in Table III is really irrelevant to the present study. At the time when it was obtained the patient had almost complete pyloric obstruction which was relieved by gastroenterostomy before the second study was made. The first curve illustrates the small and delayed hyperglycemia that results when absorption of sugar from the alimentary tract is greatly retarded or prevented.

SUMMARY.

Cutaneous and venous blood sugar values have been determined simultaneously before and at half hour intervals after the ingestion of 50 gm. of glucose or a mixed breakfast containing from 75 to 100 gm. of carbohydrate in normal subjects and patients with

[1] These curves only are employed because they are the only normal curves that were prolonged to the 2 hour point. Both the liver curves and this normal series were determined by the same individual, MF.

tract because of pyloric obstruction. This observation is omitted in the calculation of averages.
† Venous blood could not be obtained because of the absence of suitable veins.

diseases of the liver. Similar determinations on normal individuals in the postabsorptive state have been made before and after the administration of 10 units of insulin.

The characteristics of the normal arterial (cutaneous) and venous blood sugar after 50 gm. of glucose or a mixed breakfast are indistinguishable and can be summarized as follows:

1. In the postabsorptive state there is usually little or no difference between cutaneous and venous sugars; but positive (arterial > venous) differences of moderate degree occur with reasonable frequency, and negative differences of significant magnitude are occasionally observed.

2. At 30 minutes a definite arterial hyperglycemia is established, which usually still exists at the end of an hour. It is usually higher at 30 than at 60 minutes. In two-thirds of the experiments the cutaneous blood sugar had returned within the normal fasting range at the end of 90 minutes.

3. The venous sugar never rose as high as the arterial. Hence a definite positive arterial-venous difference, varying in magnitude from 6 to 50 mg., was detected in every instance. This was sometimes absent at either the 30 or 60 minute interval, but never at both. It was usually maximal at the point of greatest hyperglycemia. It was found even when cutaneous or venous sugar or both showed minimal rises. It had often disappeared by the end of 90 minutes and, indeed, at this time negative differences of significant or even considerable size were sometimes observed.

Insulin (10 units) when given to a normal individual in the postabsorptive state results in hypoglycemia which is usually most marked at the 60 minute period. The appearance of a distinct and significant positive arterial-venous difference can usually be demonstrated. During the recovery definite negative differences were demonstrated in one subject.

In severe liver disease an excessive or prolonged hyperglycemia can usually be demonstrated after 50 gm. of glucose. All the subjects investigated, however, developed a definite positive arterial-venous difference.

The bearing of these observations on clinical and physiological problems has been discussed briefly.

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PROTOCOLS.

H. M.—Male, married, age 38. 6 weeks earlier patient had abdominal distress and clay-colored stools for a short period; 3 weeks after this a short course of fever. Jaundice commenced 2 weeks before the sugar curve was made and was beginning to clear at the time the latter was made, but was still distinctly evident. Van den Bergh and bromsulfalein tests were within normal limits. No glycosuria before or during test. *Diagnosis:* Catarrhal jaundice.

A. L.—Male, brass worker, age 29. Gastrointestinal symptoms of 2 weeks duration with moderate jaundice which had developed a few days later. Van den Bergh test = 14 units; bromsulfalein = 40 per cent retained at the end of 30 minutes. No glycosuria before or during test. *Diagnosis:* Catarrhal jaundice.

C. P.—Male, age 70. Gastrointestinal symptoms of 6 weeks duration; jaundice and high colored urine for 2 weeks. At the time of the first study the patient had definite symptoms of pyloric obstruction. Van den Bergh

test = 22 units; bromsulfalein = 25 per cent retained at the end of 30 minutes. Glycosuria before, but none after test. The second study was made 3 weeks after an operation at which carcinoma of the stomach with pyloric obstruction and extensive involvement of liver, gall bladder, and bile ducts was found and a gastroenterostomy performed. Jaundice persisted. Van den Bergh test = 14 units. No glycosuria before or after test. *Diagnosis:* Carcinoma of stomach with metastases to liver and biliary obstruction.

J. S.—Male, age 62. Ascites of 2 weeks duration and slight jaundice. Van den Bergh test = 7.5 units; bromsulfalein = 42 per cent retained at the end of 30 minutes. No glycosuria before or after test. *Diagnosis:* Portal cirrhosis of the liver.

J. B.—Male, age 63. Hypertension and arteriosclerotic heart disease without heart failure. Large liver. Bromsulfalein = 12 per cent retained at the end of 43 minutes. No glycosuria before or after test.

M.—Male, age 28. Gastrointestinal symptoms and hematemesis of 10 days duration. Marked jaundice and clay-colored stools. Liver moderately enlarged. Van den Bergh test = 25 units; bromsulfalein = 45 per cent retained at the end of 30 minutes. No glycosuria before or after test. *Diagnosis:* Toxic hepatitis and cholangitis.

L. W.—Male, age 39. Jaundice and gastrointestinal symptoms had developed in the course of intravenous salvarsan treatment. Van den Bergh test = 11 units; bromsulfalein = 22 per cent retained at the end of 30 minutes. No glycosuria before or after test. *Diagnosis:* Arsenical hepatitis.

F. M.—Male, age 32. Developed septic temperature and moderate jaundice after appendectomy. On a number of occasions slight glycosuria had been observed, although no sugar was found in the urine before or after the sugar curve was made. *Diagnosis:* Pylephlebitis (?).

DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION.

XIX. DOES COPPER SUPPLEMENT VITAMIN B* FOR LACTATION?†

By BARNETT SURE.

With the Technical Assistance of Dorothy J. Walker,

(From the Laboratory of Agricultural Chemistry, University of Arkansas, Fayetteville.)

and the Cooperation of E. H. Stuart.

(From the Research Laboratories of Eli Lilly and Company, Indianapolis.)

(Received for publication, August 27, 1928.)

In pursuing further the problem of the vitamin B requirements for lactation it has occurred to us that, since the yeast concentrates employed by us as sources of vitamin B are abundant in mineral elements, some preparations containing as much as 30 per cent ash, we may be dealing with a supplementary inorganic ion factor.

The recent discovery of Hart and coworkers (1) that copper can act as a supplement to iron in the synthesis of hemoglobin and that this element is probably the active constituent of the ash of lettuce leaves, stimulated us to investigate the possibility of copper being a limiting factor in synthetic rations used in studying vitamin B requirements for lactation. Attempts, however, to replace some of our yeast extracts with the ash equivalent to the original product resulted in absolute failure. The ash of the vitamin B concentrates was administered to nursing young as well as to lactating mothers separately from the ration, but without any response. Of course, the next step would have been to feed a

* In this paper the term "vitamin B" is used to signify a combination of the thermolabile (antineuritic) and thermostable factors, both of which are necessary for growth.

† Research paper No. 68, Journal Series, University of Arkansas. Aided by a grant from Eli Lilly and Company, Indianapolis.

TABLE I.

Lactation Record of Female 4772 (First Litter, Six Young, Mother Given Six to Rear) Transferred from Stock Diet 1 on Date of Birth of Litter to Ration 1009.

Minimum Daily Vitamin B Requirement for Growth of Her Nursing Young (Yeast Concentrate P-19803).*

Date.	Female 4772.	Litter.	Food consumed during previous 24 hrs.	Date.	Female 4772.	Litter.	Food consumed during previous 24 hrs.
July, 1927	*gm.*	*gm.*	*gm.*	*Aug., 1927*	*gm.*	*gm.*	*gm.*
19	282[1]			7	188[6]	135	20
20	242	34		8	184	140	17
21	232	39	3	9	178[7]	143	20
22	240	41	12	10	185	156[15]	26
23	240	46	11	11	183	166	27
24	238	54	4	12	178[8]	162	23
25	218[2]	62	8	13	180	164	23
26	224	69	15	14	176[9]	164	22
27	222	76	13	15	182	170	28
28	210[3]	79	14	16	186	176	21
29	214	90	15	17	183	178	25
30	212	98	17	18	184	183	31
31	208	106	14	19	178[10]	184	24
Aug.				20	182	192	30
1	200	112	16	21	185[11]	193	32
2	194	117	15	22	186[12]	198	26
3	192	122	17	23	188	222	30
4	196	128[11]	18	24	186[13]	221	32
5	188[4]	133	14	25	186	236	40
6	188[5]	133	13	26	194	239[16]	26

* For method of preparation, see text.

[1] 10 mg. of Yeast Concentrate P-19803 to mother daily.

[2] 5 mg. to mother; 20 mg. to litter daily.

[3] 5 mg. to mother; 30 mg. to litter daily.

[4] 10 mg. to mother; 25 mg. to litter daily.

[5] 10 mg. to mother; 30 mg. to litter daily.

[6] 10 mg. to mother; 36 mg. to litter daily.

[7] 10 mg. to mother; 42 mg. to litter daily.

[8] 10 mg. to mother; 54 mg. to litter daily.

[9] 10 mg to mother; 60 mg. to litter daily.

[10] 10 mg. to mother; 72 mg. to litter daily.

[11] 10 mg. to mother; 78 mg. to litter daily.

[12] 10 mg. to mother; 84 mg. to litter daily.

[13] Administered yeast concentrate, dried overnight in a phosphorus pentoxide desiccator, in same dosage distributed between mother and litter.

[14] Young opened their eyes.

[15] Young eating.

[16] Young weaned.

vitamin B product free from ash, but since the possibility of obtaining such a preparation seems quite remote at present, we were obliged to resort to another method of experimental procedure. The method of experimental inquiry employed consisted of the application of the method reported previously (2). Details of the method, describing a vitamin B unit for nursing young, will appear elsewhere (3), but in order to demonstrate the character of results we secured by supplementing some of our yeast concentrates with copper administrations to lactating mothers and to nursing young, one illustrative table is submitted (Table I). The modified biological procedure is as follows: Mothers transferred with their young, reduced to six in number, from our Stock Diet 1 (4) to vitamin B-deficient Ration 1009 (5) are allowed on date of birth of litters small amounts of concentrated vitamin B preparations to prevent rapid depletion of vitamin reserves. Vitamin therapy to the litters is begun on the 4th to 5th day of lactation; in other words, the prophylactic method is used and maintenance of nurslings is thus encountered later in lactation.

Table I illustrates a representative case of the increasing daily amounts of a yeast concentrate (prepared by adsorption on silica gel and released at various hydrogen ion concentrations) (6) necessary for growth of nursing young during various stages of the lactation period. It is quite apparent that from August 5 on, the nursing young of Female 4772 needed more than 6 mg. each of the yeast concentrate for continuous growth. A submaintenance level was reached between August 11 and 14 on a daily allowance of 7 to 9 mg. per nursling. The increase to a daily dosage of 10 mg. per young, or 1 additional mg. per nursling, was followed by appreciable growth, the response being obtained in 24 hours. Because of the hygroscopic nature of the concentrate (the hygroscopicity having been increased by frequent exposure to the air in the daily manipulation of it) we have found it difficult to weigh the material accurately between August 21 and 26, but it was determined by analysis that the product during that period carried 15 per cent moisture. It will be noted that the availability of 2 mg. more of the vitamin preparation to each nursling per day (August 24 to 26), made possible through a procedure of drying the hygroscopic vitamin B concentrated product in a phosphorus pentoxide desiccator, resulted in phenomenonal growth.

Since our biological method, tested on over 200 lactating mothers and over 1000 nursing young, proved sensitive to the extent that definite responses could be secured in 24 to 48 hours, we attempted to supplement their daily vitamin allowances with small amounts of copper. Calculated amounts of Baker's cupric sulfate, C.P., were dissolved in distilled water and made up to volume so that each 0.1 cc. contained 0.01 mg. of copper. In determining the daily dosages we have followed the technique of Hart and coworkers (1) and have allowed 0.06 to 0.24 mg. daily

TABLE II.

Effect of Administration of Copper (in Form of $CuSO_4$) to Lactating Mothers or to Nursing Young on Growth of Nurslings.

Experiment No.	Cu to lactating mother.		Cu to nursing young.			No. of administrations.	Response.
	Female No.	Amount Cu administered once daily.	Litter of female No.	No. in litter.	Amount Cu administered once daily to each nursling.		
		mg.			*mg.*		
1338			5325	6	0.01	3	None.
1342	5334	0.12				3	"
1342			5335	6	0.01	3	"
1345			5340	5	0.01	3	"
1346			5343	6	0.01	2	"
					0.005	2	"
1347	5345	0.12				1	"
		0.24				2	"
1355	5363	0.12				3	"
	5364	0.12				3	"
1356	5365	0.06				3	"

dosages to the lactating mothers. To the nursing young, weighing 22 to 30 gm. each, we have allowed 0.01 mg. daily per animal in most cases (Table II), but in a few cases have reduced the daily dosage to 0.005 mg. per young. The supplementary copper administrations were given to such young that reached a maintenance level during the latter part of lactation at which time the nurslings received approximately 60 per cent of the optimum daily administration of a vitamin B concentrate necessary for continuous growth. Table II shows that twenty-three nursing

young made no response in 48 to 72 hours. The same young, however, responded readily (in 24 hours) when the daily dosage of the concentrate was increased from 15 to 20 mg. per nursling per day. A further response was obtained when the daily dosage to each young was increased to 25 mg. Five lactating mothers showed no response to administrations of 0.06 to 0.24 mg. of copper per animal per day, in addition to a daily dosage of 150 to 300 mg. of the same concentrate allowed the nursing young. The maximum daily dosage of this concentrate found necessary to wean a litter of six young was 150 mg.

We are also showing in this paper one illustration of a series of experiments completed last summer on the potency of head lettuce as a source of vitamin B for lactation.[1] Chart I shows clearly that 30 gm. of fresh head lettuce, administered daily, separately from the ration, to the lactating mother at the point of depletion of her vitamin reserves, is followed by complete failure in growth of the nursing young. For brevity we have deleted the daily growth record of the lactating mothers and the daily food consumption data and are showing graphically only the character of growth of the nursing young. The amount of lettuce we have furnished our animals daily was the maximum they would consume in addition to their basal diet, but no response in lactation was obtained.

If copper is essential for lactation and for the growth of nursing young, it must have been furnished by the lower levels of our vitamin B concentrate before maintenance was reached during the later stages of lactation. At least, additional increments of growth could not be secured by copper administrations in dosages which Hart and coworkers found effectual in the synthesis of hemoglobin and also for growth in rats that have become anemic on an exclusively whole milk diet. On the other hand, when the daily dosage of our vitamin concentrate was increased we never failed to receive a response in the growth of nursing young in as short an interval as 24 hours.

[1] The lettuce experiments were carried out with the assistance of Miss Mildred Cummings, a senior student in the Department of Home Economics, during the year 1927.

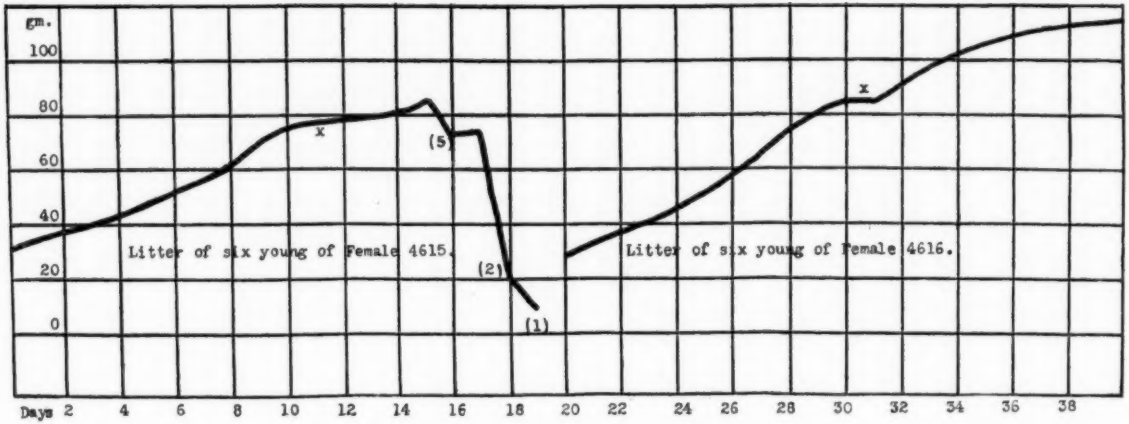

CHART I. Growth of nursing young of lactating Mothers 4615 and 4616, transferred on date of birth of litters from Stock Diet 1 to vitamin B-deficient Ration 1009. The composition of Ration 1009 is as follows: casein (purified) 20, agar-agar 2, Salts 185* 4, butter fat 5, dextrin 69. At point x mothers were allowed 30 gm. of fresh head lettuce daily. The numbers in parentheses indicate how many live young were left in the litter of six.

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.

XUM

SUMMARY.

Copper was found to have no supplementary value to a vitamin B concentrate for lactation.

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XUM

DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION.

XX. A DIFFERENTIATION OF THE VITAMIN B COMPLEX IN RICE POLISHINGS AS EVIDENCED IN STUDIES OF LACTATION.*

By BARNETT SURE.

With the Technical Assistance of Dorothy J. Walker,

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and the Cooperation of E. H. Stuart.

(*From the Research Laboratories of Eli Lilly and Company, Indianapolis.*)

(Received for publication, August 27, 1923.)

During the last few years considerable evidence has been presented to the effect that the dietary substance which was heretofore known as vitamin B is a complex composed of at least two components, one which is thermolabile and the other thermostable, both of which in combination are essential for growth. The literature on the subject has been recently reviewed by Sherman and Axtmayer (1) and by Chick and Roscoe (2).

In recent communications it has been reported that the lactating rat is incapable of secreting the vitamin B complex quantitatively in the milk (3). In this paper a differentiation is made of the two dietary factors associated with the vitamin B complex as found in rice polishings, and experimental evidence is submitted showing that the lactating animal is inefficient in secreting both the stable and the labile factors in the milk.

The experimental procedure was to transfer lactating mothers from our colony Stock Diet 1 (4) to vitamin B-deficient Ration 1009 (5) on the date of the birth of the litters. At the point of depletion of vitamin reserves of the mother the ration was changed, rice polishings replacing an equivalent amount of dextrin in the

* Research paper No. 89, Journal Series, University of Arkansas. Aided by a grant from Eli Lilly and Company, Indianapolis.

XIIM

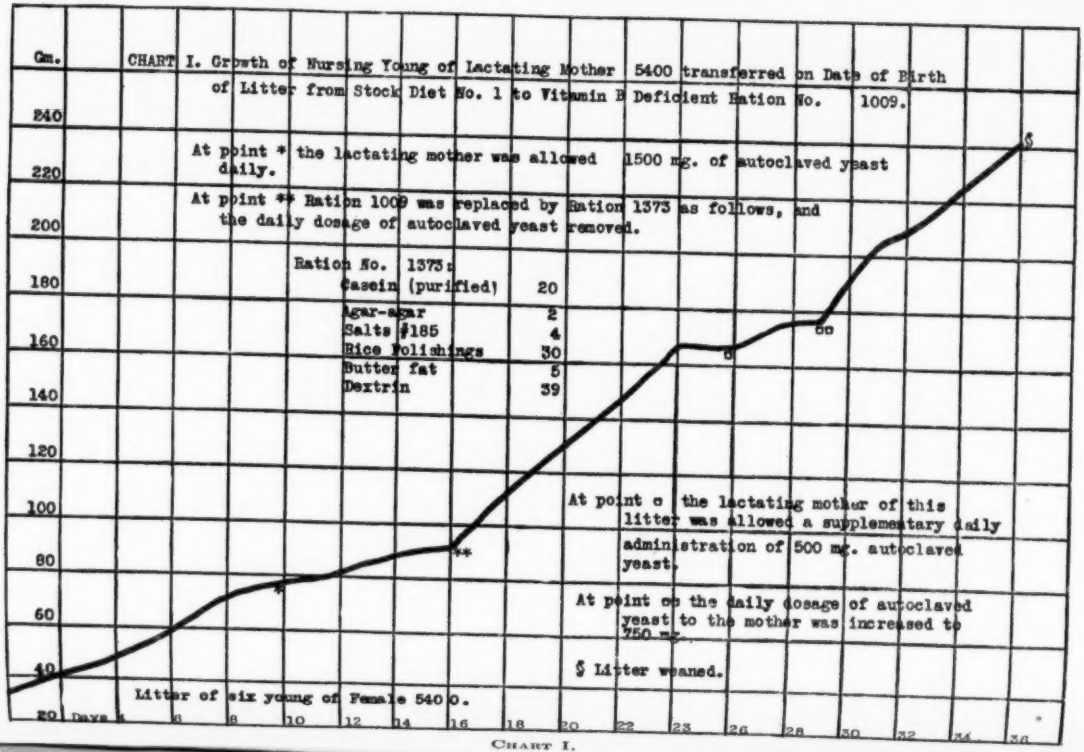
Gm.
CHART I. Growth of Nursing Young of Lactating Mother 5400 transferred on Date of Birth of Litter from Stock Diet No. 1 to Vitamin B Deficient Ration No. 1009.
At point * the lactating mother was allowed 1500 mg. of autoclaved yeast daily.
At point ** Ration 1009 was replaced by Ration 1373 as follows, and the daily dosage of autoclaved yeast removed.
Ration No. 1373:
Casein (purified) 20
Agar-agar 2
Salts #185 4
Rice Polishings 30
Butter fat 5
Dextrin 39
At point a the lactating mother of this litter was allowed a supplementary daily administration of 500 mg. autoclaved yeast.
At point aa the daily dosage of autoclaved yeast to the mother was increased to 750 mg.
$ Litter weaned.
Litter of six young of Female 5400.
240
220
200
180
160
140
120
100
80
60
40
20
Days
6
8
10
12
14
16
18
20
22
23
26
28
30
32
34
36

CHART I.

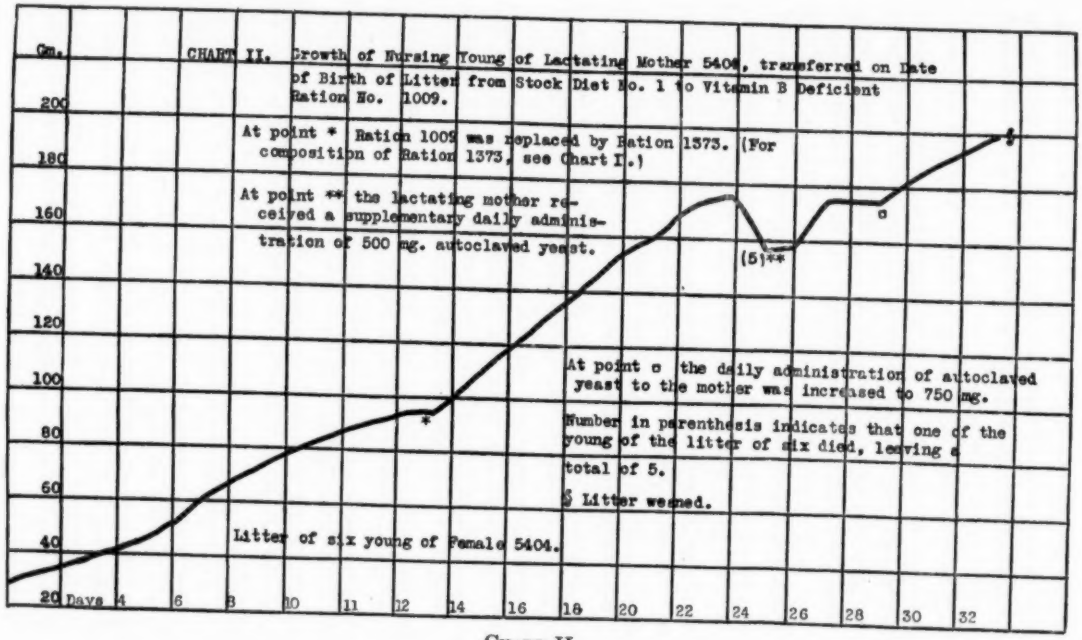
Gm.
200
180
160
140
120
100
80
60
40
20
Days 4 6 8 10 11 12 14 16 18 20 22 24 26 28 30 32
CHART II. Growth of Nursing Young of Lactating Mother 5404, transferred on Date of Birth of Litter from Stock Diet No. 1 to Vitamin B Deficient Ration No. 1009.
At point * Ration 1009 was replaced by Ration 1373. (For composition of Ration 1373, see Chart I.)
At point ** the lactating mother received a supplementary daily administration of 500 mg. autoclaved yeast.
At point c the daily administration of autoclaved yeast to the mother was increased to 750 mg.
Number in parenthesis indicates that one of the young of the litter of six died, leaving a total of 5.
$ Litter weaned.
Litter of six young of Female 5404.
(5)**
*
c
$

CHART II.

diet. For brevity we are deleting daily records of weight of mothers as well as daily food consumption data, and are showing graphically only representative cases of our results as evidenced by growth of nursing litters.

Chart I shows that at point * before the ration was changed, a slight response was secured by the supplementary daily administration of 1500 mg. of yeast autoclaved for 5 hours at 15 to 18 pounds pressure, but marked response followed at point ** when the autoclaved yeast was removed and the ration changed, 30 per cent rice polishings replacing an equivalent amount of dextrin in the diet. Such procedure resulted in phenomenal growth in the nursing young for the subsequent 8 days, and another maintenance curve in the litter was reached on the 24th day of lactation. On the 26th day, at point □ the lactating mother received a supplementary daily allowance of 500 mg. of autoclaved yeast, which resulted in growth of the nurslings for 48 hours, but another plateau was encountered the subsequent 24 hours. On the 29th day, at point □ □ the increase of the daily dosage of autoclaved yeast to the mother to 750 mg. stimulated excellent growth in the litter for the rest of the 7 day experimental period.

Chart II shows the results of a similar experiment. In this case the point of depletion of the stable factor in the lactating mother could not be readily anticipated, and one of the six nurslings died, undoubtedly for lack of an optimum amount of the thermostable substance. Following the death of this nursing young the supplementary daily administration of autoclaved yeast to the mother was begun. The responses to the 500 and 750 mg. daily allowances of autoclaved yeast to the lactating mother, judging by the character of growth of the nursing young, are apparent.

The results shown in Charts I and II are typical of six additional experiments and show that rice polishings fed at a 30 per cent plane of intake provide an abundance of the antineuritic (labile) factor for lactation, but an insufficiency of the stable factor, also a necessary constituent of milk for continuous growth of nursing young.

Charts III and IV show that rice polishings, fed at a 20 per cent level, do not even furnish enough of the antineuritic factor for growth of the nursing young, since as much as 1500 mg. daily of autoclaved yeast failed as a supplement to the lactating mothers.

Chart IV reveals that, even when the preventive method is employed by administering the supplementary allowances of autoclaved yeast to the mother before depletion of reserves of the stable

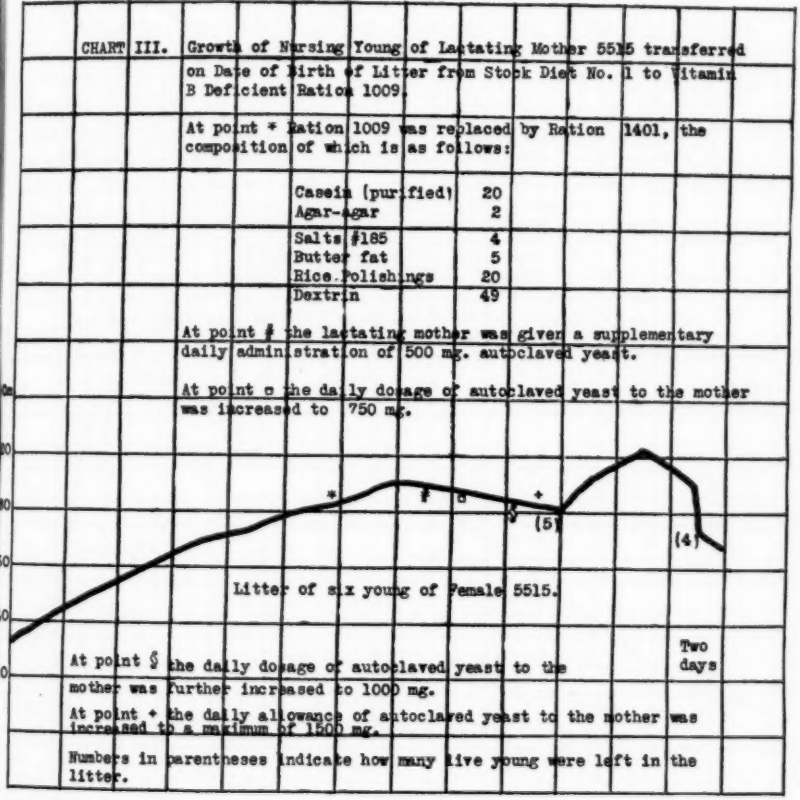
CHART III. Growth of Nursing Young of Lactating Mother 5515 transferred on Date of Birth of Litter from Stock Diet No. 1 to Vitamin B Deficient Ration 1009.
At point * Ration 1009 was replaced by Ration 1401, the composition of which is as follows:
Casein (purified) 20
Agar-agar 2
Salts #185 4
Butter fat 5
Rice Polishings 20
Dextrin 49
At point # the lactating mother was given a supplementary daily administration of 500 mg. autoclaved yeast.
At point o the daily dosage of autoclaved yeast to the mother was increased to 750 mg.
Litter of six young of Female 5515.
At point § the daily dosage of autoclaved yeast to the mother was further increased to 1000 mg.
At point + the daily allowance of autoclaved yeast to the mother was increased to a maximum of 1500 mg.
Numbers in parentheses indicate how many live young were left in the litter.
Two days

CHART III.

factor, we cannot circumvent subsequent infant mortality, because of the insufficient amounts of the antineuritic (labile) factor supplied by the lower level of rice polishings.

Inefficiency of the Lactating Mother in Secreting Stable Factor in Milk.

We have observed in a number of cases that, although the lactating mothers responded to the administrations of autoclaved yeast supplementing a ration containing 30 per cent rice polish-

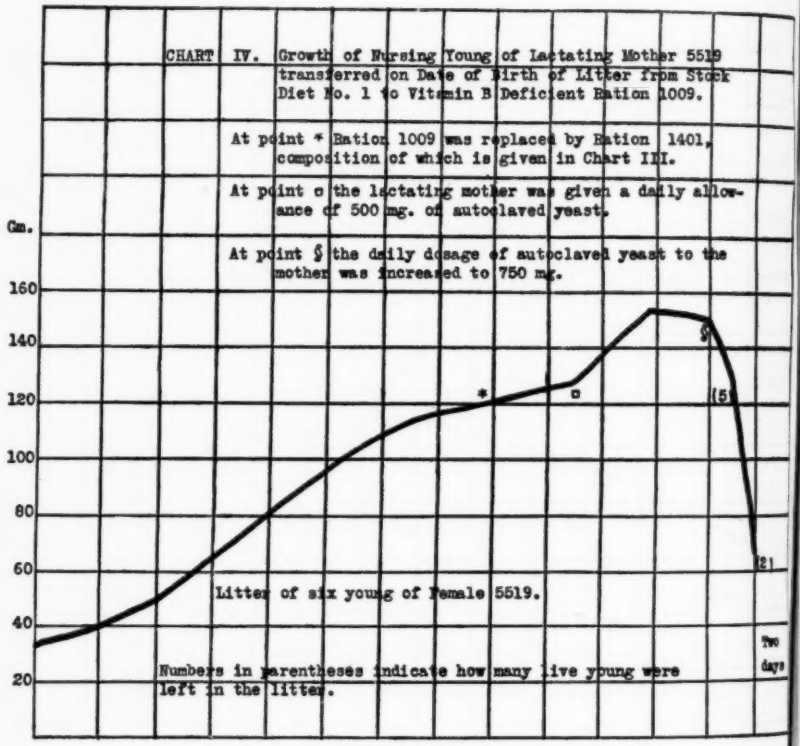
CHART IV. Growth of Nursing Young of Lactating Mother 5519 transferred on Date of Birth of Litter from Stock Diet No. 1 to Vitamin B Deficient Ration 1009.
At point * Ration 1009 was replaced by Ration 1401, composition of which is given in Chart III.
At point o the lactating mother was given a daily allowance of 500 mg. of autoclaved yeast.
At point § the daily dosage of autoclaved yeast to the mother was increased to 750 mg.
Gm.
160
140
120
100
80
60
40
20
*
o
§
(5)
(2)
Litter of six young of Female 5519.
Numbers in parentheses indicate how many live young were left in the litter.
Two days

CHART IV.

ings, they were rearing their young at a subnormal rate. All attempts to distribute a daily dosage of 500 to 750 mg. between lactating mothers and nursing young resulted in failure in four litters, because the nursing young absolutely refused to consume

CHART V. Growth of Nursing young of Lactating Mother 5471 transferred on Date of Birth of Litter from Stock Diet No. 1 to Vitamin B Deficient Ration 1009.

At point * Ration 1009 was replaced by Ration 1373, composition of which is given in Chart I.

At point ** the lactating mother was given a supplementary daily administration of 500 mg. autoclaved yeast.

At point # the daily dosage of autoclaved yeast to the mother was increased to 750 mg.

At point o the daily dosage of 750 mg. of autoclaved yeast was distributed between mother and young, allowing 250 mg. to the mother and 500 mg. to the litter of 5 young.

Litter of six of Female 5471.

$ Litter weaned.

Number in parenthesis indicates that one of the young of the litter of six died, leaving 5 nurslings.

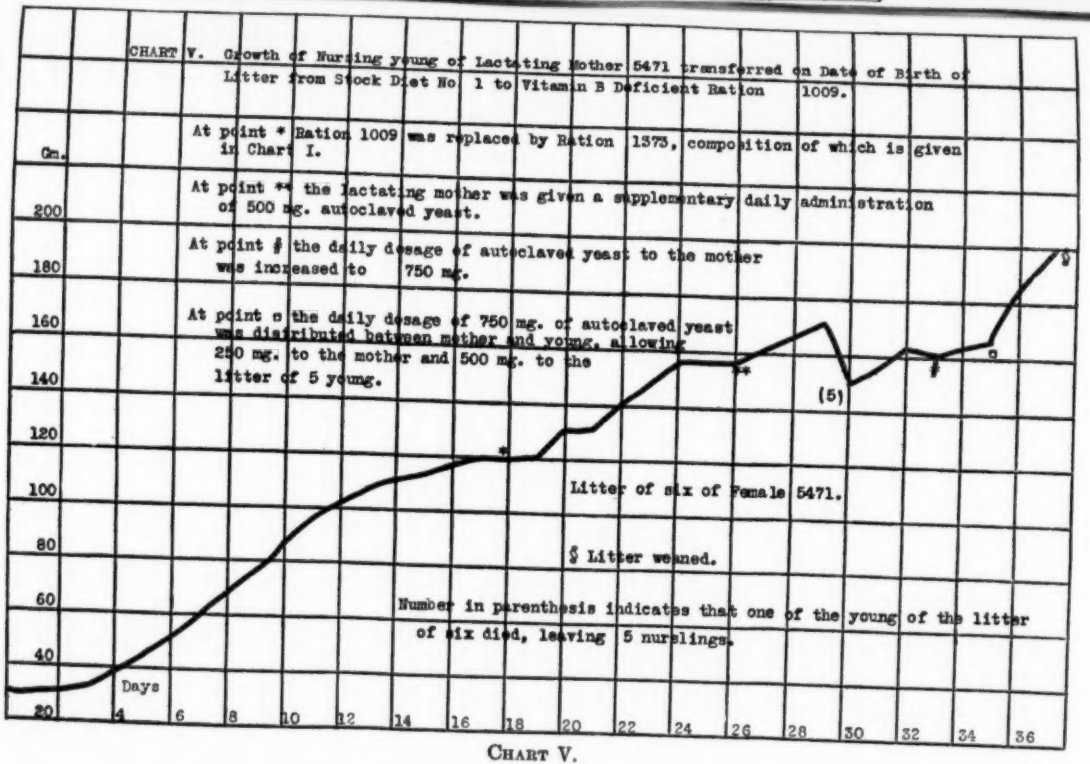

CHART V.

the autoclaved product. This we were, however, later able to accomplish with two litters. Chart V shows the results of the character of response of one of those litters. Between the 32nd and 35th day of lactation the remaining litter of five young of Female 5471 gained 5 gm. when the lactating mother was receiving a supplementary daily administration of 750 mg. of autoclaved yeast. On the 35th day of lactation the 750 mg. daily allowance of autoclaved yeast was distributed, allowing 250 mg. to the mother and 500 mg. to the litter, or 100 mg. to each nursling, and during the subsequent 48 hours the litter gained 35 gm.

Another litter responded in a similar manner when during the latter part of lactation the 500 mg. daily dosage of autoclaved yeast, allowed the lactating mother, was given to her litter.

In addition to the two experiments just cited, we have obtained identical results with four more litters on a somewhat modified dietary régime. In these cases the mother and young received a distribution of a vitamin concentrate prepared from an alcoholic extract of rice polishings by adsorption on fullers' earth. In this way we have provided an abundance of the antineuritic factor to the mother and nursing young. The mothers received in addition a daily dosage of 1500 mg. of autoclaved yeast. When, during the later part of lactation the litters showed prolonged maintenance, the daily portion of autoclaved yeast given the mother was distributed, allowing the nursing young the greater part of the dosage. In every case such procedure was followed by accelerated growth of the litters. From the above experiments we conclude that the lactating rat is incapable of secreting the stable, growth-promoting factor, associated with the vitamin B complex, quantitatively in the milk.

Inefficiency of the Lactating Mother in Secreting Antineuritic (Labile) Factor in Milk.

In proceeding with the quantitative study of the secretion of the antineuritic (labile) factor in the milk we took advantage of the fact that in the preparation of various vitamin concentrates we had access to one product obtained from yeast which was deficient in the antineuritic but abundant in the stable factor. We also had an alcoholic extract from rice polishings which, although unusually potent in the antineuritic, was markedly deficient in the

stable substance. After a number of litters reached a maintenance curve during the later part of lactation on a distribution of 130 mg. daily of our Yeast Concentrate 21 (abundant in the stable, but deficient in the antineuritic factor) between mother and young, allowing daily 10 mg. to the mother and 120 mg. to litters of six young, each mother received in addition 6 drops of a concentrated alcoholic extract of rice polishings. The responses in 48 hours were insignificant. The litters either continued to maintain their weight or gained 2 to 3 gm. The 6 drops of the same vitamin concentrate were then administered to the litters, 1 drop to each young daily, and in every case the young responded phenomenally. During the subsequent 2 days each litter gained as much as 30 gm. Six litters of 48 young were subjected to this experimental study and not one of these nurslings failed to respond readily to supplementary administrations of the antineuritic factor furnished by the alcoholic extract of rice polishings, whereas all the mothers failed to secrete this substance in the milk in sufficient amounts to meet the requirements of the nursing young for growth. It is apparent, then, from these experiments that the lactating rat is unable to secrete quantitatively in the milk the antineuritic (labile) growth-promoting factor as well as the stable, growth-promoting substance.

DISCUSSION.

From our food consumption records it has become apparent that 7 gm. of rice polishings per lactating rat per day furnish an abundance of the antineuritic (labile) factor, but an inadequate amount of the stable factor for lactation. On the other hand, 2.3 gm. of rice polishings per animal per day have supplied an abundance of both factors in rats for growth to the extent that young animals (whose stores of vitamin reserves of both factors had been entirely depleted when the experiments were begun at weaning) trebled their weaning weights in 58 days. At this writing we have based such findings on twenty-four animals. It is clear, then, that the requirements of the stable factor for lactation are more than 3 times that necessary for growth. The requirements of the antineuritic (labile) substance for lactation are also considerably greater than for growth, since we have found that a daily allowance of 5 gm. of rice polishings (as a source of the antineuritic

factor) per lactating rat per day proved inadequate for milk secretion, whereas a daily amount of 2.3 gm. of rice polishings per animal per day has so far been excellent for growth for as long as 2 months. We are continuing these growth experiments for longer periods of time.

Our findings are not in harmony with the recent report of Evans and Burr (6) that "lactation delinquency is due to inadequate antineuritic vitamin, and in no degree to inadequate growth-promoting vitamin B," since our conclusions are that larger amounts of both factors are essential for lactation than for growth. The references of Evans and Burr (6) and Hunt (7) to the antineuritic as contrasted with the growth-promoting substance are misleading, since both the antineuritic and stable factors are growth-promoting. The responses to either of these dietary factors, associated with the vitamin B complex, in growth of nursing young have been demonstrated in the experimental evidence presented in this communication.

Nomenclature of Dietary Factors Associated with Vitamin B Complex.

A differentiation of the fat-soluble vitamins A and D in cod liver oil was made by McCollum and coworkers (8), mainly on the grounds of thermostability. We are, therefore, justified from the extensive evidence recently accumulated that the dietary complex, which McCollum and Kennedy in 1915 termed water-soluble B (9) is also made up of two specific vitamins. The recent work of Chick and Roscoe (2) confirms the original observations of Goldberger and associates (10) that a deficiency of the vitamin, which is thermostable, produces in the rat a dermatitis quite similar to that observed in human pellagra. It also now seems apparent that Eijkman, when in 1897 he produced the classical demonstration of polyneuritis in animals (11), was dealing with a deficiency of the antineuritic, thermolabile vitamin.

The English biochemists have provisionally adopted the nomenclature B_1 and B_2 for the labile and stable vitamins respectively (2). Such nomenclature is objectionable because it still leaves the impression that we are dealing with two phases of one rather than with two separate vitamins. Sherman and Axtmayer (1) have, therefore, suggested the adoption of letters F and G to

represent the labile and stable vitamins respectively, and that the term vitamin B should represent the combination of vitamins F and G. The objection to this nomenclature is that no special physiological function is assigned to Eijkman's classical antiberiberi vitamin, which has for the last 13 years been known as vitamin B, and the universal recognition of which has been largely responsible for the stimulation of vitamin research. I am, therefore, in accord with the recent suggestion of Hogan and Hunter (12) that we retain the letter B for the thermolabile, antiberiberi vitamin of Eijkman, and add the letter F to represent the thermostable, antipellagra vitamin of Goldberger and associates, with the understanding that both of these vitamins are growth-promoting in the sense that fat-soluble vitamins A and D are both growth-promoting.

SUMMARY.

In accordance with the nomenclature suggested in the text for the dietary factors associated with the vitamin B complex, it is concluded from the results reported in this paper that rice polishings contain in addition to large amounts of vitamin B an appreciable amount of vitamin F for growth, but from the standpoint of lactation rice polishings, while abundant in vitamin B, proved deficient in vitamin F.

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